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Contents

No. 1 (*issued* 26 January 1961)

PAGE

A Study of Radiosensitive and Radioresistant Mutants of *Escherichia coli* strain B BY R. F. HILL and E. SIMSON 1

The Measurement of Bacterial Viabilities by Slide Culture BY J. R. POSTGATE, J. E. CRUMPTON and J. R. HUNTER 15

The Effect of Asparagine on the Growth of a Gram-positive Coccus BY E. I. GARVIE, M. E. GREGORY and L. A. MABBITT 25

Comparative Carbohydrate Metabolism and a Proposal for a Phylogenetic Relationship of the Acetic Acid Bacteria BY J. DE LEY . . . 31

The Effect of Monovalent Cations on the Inhibition of Yeast Metabolism by Nystatin BY F. MARINI, P. ARNOW and J. O. LAMPEN . . . 51

Morphological and Biological Properties of a Strain of Chromogenic Acid-fast Bacteria and of its Phage-immune Variant BY E. MANKIEWICZ . . 63

The Effect of Biotin Deficiency on the Synthesis of Nucleic Acids and Protein by *Saccharomyces cerevisiae* BY F. AHMAD, A. H. ROSE and N. K. GARG 69

The Cultivation of D-Galactosamine-metabolizing Lactic Acid Bacteria BY H. BLUMENTHAL, T. SHIOTA, M. P. McCANN and M. N. DISRAELY . 81

Reversible Bicarbonate-induced Enzyme Activity and the Point of no Return during Morphogenesis in Blastocladiella BY J. S. LOVETT and E. C. CANTINO 87

The Endogenous Metabolism of *Euglena gracilis* BY W. F. DANFORTH and B. W. WILSON 95

The Fixation of Tetanus Toxin by Ganglioside BY W. E. VAN HEYNINGEN and P. A. MILLER 107

The Relation between the Tetanus Toxin-fixing and Influenza Virus-inhibiting Properties of Ganglioside BY A. W. BERNHEIMER and W. E. VAN HEYNINGEN 121

Further Studies on the Repression of Methionine Synthesis in *Escherichia coli* BY R. J. ROWBURY and D. D. WOODS 129

Observations on the Specificities of Extracellular Antigens of the Genera *Aeromonas* and *Serratia* BY P. V. LIU 145

A Volatile Substance Controlling Early Stages of Zygospore Formation in *Rhizopus sexualis* BY P. M. HEPDEN and L. E. HAWKER . . . 155

No. 2 (*issued* 10 February 1961)

PAGE

Toxicity of the Extracellular Phase of *Prymnesium parvum* Cultures BY J. YARIV and S. HESTRIN 165

Purification of Murray Valley Encephalitis Virus BY G. L. ADA, S. G. ANDERSON and A. ABBOT 177

A Strain of *Bacillus circulans* Capable of Growing under Highly Alkaline Conditions BY M. E. CHISLETT and D. J. KUSHNER 187

The Effect of Oxygen on Freeze-dried *Escherichia coli* BY M. B. LION and E. D. BERGMANN 191

Inter-species Change in Thiobacilli BY K. I. JOHNSTONE, M. TOWNSHEND and D. WHITE 201

Decomposition of Pyrimidines by *Nocardia corallina* BY R. D. BATT and D. D. WOODS 207

Morphogenesis of Mycoplasma and Bacterial L-form Colonies BY S. RAZIN and O. OLIVER 225

The Influence of Hydrogen-Ion Concentration and 2:4:Dinitrophenol on Orthophosphate Accumulation in *Tetrahymena pyriformis* BY R. L. CONNER, R. GOLDBERG and M. S. KORNACKER 239

A Disease of Finnock Due to *Vibrio anguillarum* BY I. W. SMITH . . 247

The Sugar Composition of Streptococcal Walls and its Relation to Haemagglutination Pattern BY W. S. L. ROBERTS and F. S. STEWART . . 253

Morphological and Biochemical Features of 'Atypical' Mycobacteria BY A. CSILLAG 261

Isolation and Classification of a New Series of Azotobacter Bacteriophages BY J. T. DUFF and O. WYSS 273

On Sporulation in Sulphate-reducing Bacteria BY the late M. E. ADAMS and J. R. POSTGATE 291

The Occurrence and Location of Teichoic Acids in Lactobacilli BY J. BADDILEY and A. L. DAVISON 295

A Mutational Alteration of the Tryptophan Synthetase of *Escherichia coli* BY F. GIBSON, M. I. GIBSON and C. YANOFSKY 301

No. 3 (*issued* 23 March 1961)

Virolysin, a Virus-induced Lysin: its Appearance and Function in the Phage-Infected Staphylococcal Cell BY D. J. RALSTON, B. BAER, M. LIEBERMAN and A. P. KRUEGER 313

A Protein Factor in the Nutrition of *Paramecium caudatum* BY D. M. LILLY and R. C. KLOSEK 327

Some Aspects of the Genetics of Methionineless Mutants of *Salmonella typhimurium* BY D. A. SMITH 335

PAGE

Phenotypic Alterations Associated with the Bacteriophage Carrier State of *Shigella dysenteriae* BY KOIBONG LI, L. BARKSDALE and L. GARMISE 355

Stimulation of Streptomycin-Resistant Bacteria in the Rhizosphere of Leguminous Plants BY M. E. BROWN 369

Chemical and Metabolic Properties of Various Elements Found in Cultures of a Stable Proteus L Form BY C. WEIBULL and H. BECKMAN . . 379

The Effect of Temperature on the Production by Coagulase-positive Staphylococci of Staphylokinase and other Extracellular Substances BY C. BUNGAY 393

Experimental Conditions for Nitrate Reduction by Certain Strains of the Genus *Lactobacillus* BY M. ROGOSA 401

Purification and Properties of Neuraminidase from *Vibrio cholerae* BY G. L. ADA, E. L. FRENCH, P. E. LIND and with the technical assistance of K. CLARKE 409

Electrophoretic, Sedimentation and Diffusion Behaviour of Crystalline Neuraminidase from *Vibrio cholerae* BY J. PYE and C. C. CURTAIN . 423

Morphological Variation in *Spirillum* spp., with Observations upon the Origin of the Hyphomicrobia BY K. A. BISSET 427

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A Study of Radiosensitive and Radioresistant Mutants of *Escherichia coli* strain B

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(*Received* 16 *May* 1960)

SUMMARY

Relative differences in radiosensitivity between *Escherichia coli* strain B and the mutant strains, B/r (more resistant than B) and two types of B_s (more sensitive), are maintained with respect to inactivation by ultraviolet radiation, X-rays and decay of incorporated ^{32}P. The differences cannot be ascribed to variations in number of nuclei or in content of DNA or RNA. The effectiveness of various post-irradiation and post-decay treatments in preventing inactivation varies for each strain. With the exception of reactivation by light, strain B/r is less reactivable than B and B_s.

The strains do not differ in their rates of mutation to resistance to bacteriophage T_1. However, the rates of mutation of the B_s mutants to the first step of furacin-resistance are considerably lower than the rate for the parent strain B. From one of the B_s mutants, new strains were obtained whose properties are mixtures of those of B and B/r.

INTRODUCTION

Among bacteria *Escherichia coli* strain B and its radioresistant mutant B/r have been frequently employed in studies concerned either with the mechanism of inactivation by radiation or with the genetic basis of radiation sensitivity (Witkin, 1947). It has been established, for instance, that B and B/r differ not only in the lethal effect of X-rays and ultraviolet radiation but also in such related properties as the shapes of their radiation survival curves, the severity of their cytological response to irradiation and their reaction to various post-irradiation treatments (Payne, Hartman, Mudd & Phillips, 1956; Anderson, 1951; Roberts & Aldous, 1949). With respect to the genetic aspects, it appears that B and B/r differ by a single gene.

A recent report described briefly the isolation of another radiological mutant of *Escherichia coli* strain B—called B_s—which is more radiosensitive (Hill, 1958*a*). It is of interest both to microbial radiobiology and to microbial genetics to inquire which properties other than radiosensitivity are affected by these contrasting mutations in strain B. The present paper will give the results of an investigation designed to elucidate the basis for differences in radio-sensitivity by studying the following points:

(1) The relationship between the type of mutation and the effectiveness of various post-irradiation treatments in preventing lethality.

(2) The nuclear multiplicity, DNA and RNA contents of strains B, B/r and B_s.

(3) The relative response to decay of incorporated ^{32}P, another lethal agent which is reported to act directly on the DNA of bacteria (Fuerst & Stent, 1956).

(4) The relative response to the antibiotic furacin. Mutation to the first step of furacin resistance is invariably accompanied by increased radioresistance and vice versa (Szybalski & Nelson, 1954). The effect of mutation to radiosensitivity on furacin sensitivity was therefore studied.

(5) The relative ability of strains B and B_s to mutate spontaneously to the B/r form. In order to compare this with the relative ability to mutate at other genetic loci, the spontaneous rates of mutation to resistance to bacteriophage T_1 were also determined.

METHODS

Bacterial strains. Strains B, B/r and B_{s-1} (this strain was called B_s in the previous report) have been described previously (Hill, 1958*a*). Strain B_{s-2} was isolated more recently in the same way as B_{s-1}. Strain B_{s-1} was found among twelve colonies of strain B originating from survivors of a high dose of u.v. radiation (5×10^{-4} survival); B_{s-2} among 100 such colonies. The frequency of B_s mutants after u.v. irradiation is therefore of the order of 1–2 % and comparable to the frequency of B/r mutants after u.v. irradiation (Witkin, 1947).

Ultraviolet irradiation. The experimental set-up has been described previously (Hill & Rossi, 1952). The strains were grown by aeration in 0·8 % nutrient broth for 24 hr. and then diluted 1/50 into physiological saline. Separate 3 ml. samples were irradiated with each dose (unfractionated irradiation). One tenth ml. samples of appropriate dilutions were plated by the agar layer technique commonly used for bacteriophage assays (Adams, 1950). Colonies were counted after 24 hr. incubation at 37°.

For the diagnosis of colonies as consisting of cells of B, B/r or B_s, a modification of Witkin's method was used (1947). Each colony was suspended in 1 ml. broth, diluted further in broth, grown to saturation and subcultured twice more. A loopful was streaked over a small area on a nutrient agar plate, which was then u.v. irradiated with a dose sufficient for 10 % survival of strain B. After 3 hr. incubation at 37° the spread areas were examined microscopically. Cells of strain B show 'snake' formation; cells of B/r form short filaments and are collected into microcolonies; the cells of B_{s-1} and B_{s-2} remain small, with a few short filaments appearing in areas containing B_{s-2}. After examination, the plates were re-incubated overnight and the spread areas re-examined without a microscope. Areas containing B or B/r show heavy confluent growth; those containing B_{s-2} show many small, separated colonies; those containing B_{s-1} are either blank or show at most one or two colonies. In cases of doubt, the colony was subcultured once more and given a test dose of u.v. radiation, after spreading an appropriate dilution on a nutrient agar plate. The test dose (20 sec. of u.v. under these conditions) gave the following surviving fractions for our bacterial stocks: B_{s-1}—1×10^{-7}, B_{s-2}—1×10^{-5}, B—1×10^{-2}, B/r—8×10^{-1}.

X-irradiation. The X-ray technique has been described (Hill, 1958*b*). Cultures were grown by aeration in nutrient broth for 24 hr. and 5 ml. undiluted aliquots irradiated.

Inactivation by decay of incorporated ^{32}P. This procedure has also been described (Fuerst & Stent, 1956; Hill, 1960). Each culture was grown for 1½ hr. in non-radioactive medium so that the organisms were in the logarithmic phase. They were then diluted 1/10 into ^{32}P-containing medium and growth continued for another 4 hr. The cultures were diluted into cold non-radioactive medium and samples frozen and stored at −78°. All four strains were grown in samples of the same batch of ^{32}P-labelled medium on the same day.

'*Reactivation*'. Heat reactivation (HR) was determined by preparing two sets of plates containing spread bacteria and incubating one set at 37° and the other at 45°. The numbers of colonies appearing after 19–22 hr. were then compared. In the experiments involving reactivation by the plating medium (PMR), the numbers of colonies appearing on nutrient broth agar plates were compared with those on synthetic M9-agar plates (Dulbecco, 1950). Photoreactivation (PHTR) was obtained in the following manner: Two ml. of the u.v.-irradiated cultures or of radioactive samples (after dilution into physiological saline) were put into test tubes supported in a specially constructed stainless-steel bath. (This apparatus was built by the Laboratory Glass and Instrument Corp., 514 W. 147 St, N.Y.C., N.Y. and was generously lent to us by S.A. Ellison.) One face of the bath consisted of quartz. The bath contained a clear lucite inner tank filled with 0·05 M-$CuCl_2$ and a test-tube rack. The space between the outer walls of the bath and the inner tank and rack was filled with water maintained at 37° by means of a heating coil and thermoregulator. The lamp used for illumination (Hill & Rossi, 1952) was placed 15 cm. from the quartz face of the bath. After an exposure of 1 hr., the cultures were diluted appropriately and plated for colony count.

Nuclear multiplicity. Twenty-four hr. aerated broth cultures were grown and samples spread on nutrient agar plates. After 15 min. and after 2 hr. of incubation at 37°, stained impression smears were prepared (Robinow, 1944; Witkin, 1951). Photomicrographs of representative fields were then made and used to count the average number of 'nuclei' per cell.

DNA and RNA contents. These chemical analyses were performed by Dr Herman Shapiro of the Cell Chemistry Laboratory, Department of Biochemistry, Columbia University. The DNA and RNA contents of 24 hr. aerated broth cultures were determined colorimetrically by using the diphenylamine and orcinol reactions, as described by Dische (1955).

The total number of bacteria was determined by a modification of the Millipore filter method (Ecker & Lockhart, 1959). One hundredth of a ml. of each culture was diluted into 5 ml. of physiological saline and diluted further 1/10 with 1 % picric acid. Ten ml. of the picric-acid dilution were filtered; the filtered cells were stained with 0·1 % acid fuchsin and then counted.

Inhibition by furacin. The antibiotic, furacin (5-nitro-2-furaldehydesemicarbazone) was obtained from the Eaton Laboratories, Norwich, N.Y. The inhibiting concentration was determined by the gradient plate technique (Szybalski, 1952).

Mutability. Spontaneous mutation rates were estimated from the average numbers of mutants appearing in series of cultures grown in parallel (Luria & Delbrück, 1943). The double-irradiation technique for the detection of B/r mutants in cultures of strain B could not be applied to cultures of B_{s-1} and B_{s-2} (Witkin, 1947). This technique is based upon the formation of snake formation by irradiated cells (Payne *et. al.* 1956). Attempts to induce snake formation by irradiating cells of B_{s-1} and B_{s-2} have been unsuccessful so far. Therefore mutants of the B/r type in cultures of B, B_{s-1} and B_{s-2} were detected indirectly by selecting for first-step furacin-resistant mutants.

In the first set of experiments involving mutation to furacin resistance, the cultures were grown in nutrient broth without aeration. One tenth ml. of each culture and of a 1/10 dilution were spread on the surface of nutrient agar plates containing 1·5 μg. furacin/ml. This concentration is inhibitory for all bacteria of strains B, B_{s-1} and B_{s-2} under our conditions and slightly less than the concentration required to inhibit the majority of bacteria of type B/r (see below). In later experiments, a pour-plate technique was used. The cultures were grown by aeration and used to determine mutation rates both to furacin resistance and to phage T_1 resistance. Cultures of strain B were diluted 1/100 and 0·1 ml. was added to a tube containing 10 ml. of nutrient agar plus 1·5 μg. furacin/ml. at 45°. The contents of the tube were then poured into a Petri dish, allowed to harden and then incubated. In the case of B_{s-1}, 1 ml. of the undiluted cultures was added to 9 ml. of the furacin-containing agar. One tenth ml. of the remainder of the cultures of each strain was then spread on plates which had been previously spread with 5×10^9 T_1 phages.

RESULTS

Survival after u.v.-irradiation, X-irradiation and ^{32}P decay

Figure 1*a* shows the u.v. survival curves of B, B_{s-1} and B_{s-2} and for comparison, the beginning of the sigmoid curve characteristic of B/r (Witkin, 1947). It can be seen that although both B_{s-1} and B_{s-2} are more radiosensitive than the parent strain B, they are not identical.

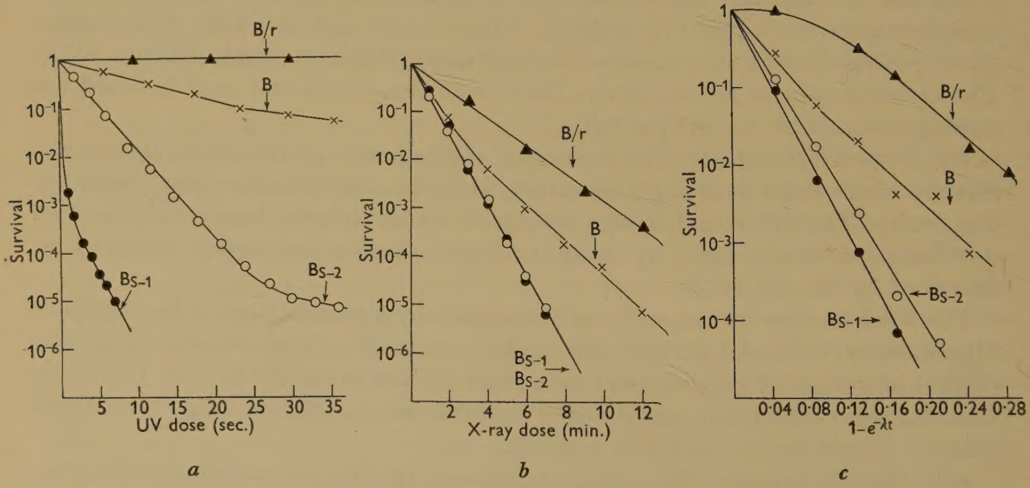

Fig. 1. Survival of *Escherichia coli* strain B and its mutants after (*a*) u.v.-irradiation, (*b*) X-irradiation and (*c*) decay of incorporated ^{32}P. The dose rates were 5·6 ergs/mm^2/sec for u.v. and 3000 roentgens/min for X-rays; the specific activity of the ^{32}P in each strain was 20 mc./mg total P.

The survival curves for B, B_{s-1} and B_{s-2} have the same shape, i.e. a decreased slope at higher doses. In the case of strain B, this change in curvature cannot be fully explained by the selective survival of B/r mutants and is usually interpreted as due to the presence of bacteria which are phenotypically but not genetically radioresistant (Cavalli & di Modrone, 1949; Gunter & Kohn, 1956; Buzzell, 1956). This situation was investigated for mutants B_{s-1} and B_{s-2} by testing 100 colonies of each stemming from cells surviving high doses, corresponding to the final portions of the curves. In the case of B_{s-2}, the final slope was about the same as the final slope for B (and B/r). In one experiment, 84 out of 100 colonies corresponding to 6×10^{-6} survival exhibited the well-defined snake formation characteristic of strain B. Two of these colonies were subjected to a test dose of u.v.; their survivals were 2·4 and 1·5 %, in agreement with the 1·9 % survival of strain B used for comparison. However, in another experiment, only 1 out of 100 colonies corresponding to 2×10^{-6} survival of B_{s-2} showed snake formation. Therefore it may be concluded that, although a form resembling strain B can be isolated after large doses of u.v. to B_{s-2} cultures, the change in curvature of the B_{s-2} survival curve cannot be wholly accounted for by the presence of this form in the unirradiated population.

In the case of B_{s-1}, the final slope of the survival curve was considerably greater than the final slopes of the curves for the other strains. Of 100 colonies corresponding to 1×10^{-5} survival, all gave a cytological response typical of B_{s-1}. Thus with B_{s-1}, as with B and B_{s-2}, the change in slope of the survival curve cannot be wholly due to a genetically resistant fraction. If the change is due to a phenotypically resistant fraction, the phenotype is not that of the presumed resistant phenotype present in either the B or B_{s-2} populations.

Although the u.v. survival curves for B_{s-1} and B_{s-2} were clearly separated, the X-ray survival curves coincided (Fig. 1*b*). Furthermore, the X-ray curves, unlike the u.v. curves, were exponentials. After X-irradiation, the survival curve for the parent B still had the shape characteristic of u.v.-irradiation, i.e. concave upward.

The survival curves corresponding to inactivation by decay of incorporated ^{32}P were also clearly separated and in the same relative positions as for u.v.-inactivation (Fig. 1*c*). It may be noted that in the case of all three inactivating agents, the survival curve for strain B has the same shape—concave upward. The curves for B_{s-1} and B_{s-2} have this shape only for u.v.-irradiation; for X-irradiation and for ^{32}P decay, the curves are exponentials. On the other hand, the curve for B/r is an exponential only for X-irradiation and is sigmoid for u.v. and ^{32}P decay (Witkin, 1947; Fuerst & Stent, 1956).

Prevention of lethal effects (‘*reactivation*’)

Figure 2 shows the effectiveness of HR, PMR and PHTR after u.v.-irradiation of the four strains. Certain differences are readily apparent. In the case of the parent strain B, all three reactivation mechanisms were equally effective. PHTR was effective for B/r but HR had no effect and PMR caused an increase in mortality after high doses of u.v. (Roberts & Aldous, 1949). In the case of the two radiosensitive mutants, all three reactivation methods were effective but not to the same extent. For both mutants, PHTR was the most efficient method. In the case of B_{s-1}, HR was more effective than PMR but the reverse was true for B_{s-2}.

Similar differences in reactivability were observed with X-irradiation and decay of incorporated ^{32}P. For example, Fig. 3 shows that after exposure to X-rays, HR was more effective for strain B than for B_{s-1}. HR was ineffective for B_{s-2} and lethal for B/r. In the case of ^{32}P decay also, HR was effective only for B (Hill, 1960) and to a smaller extent for B_{s-1}.

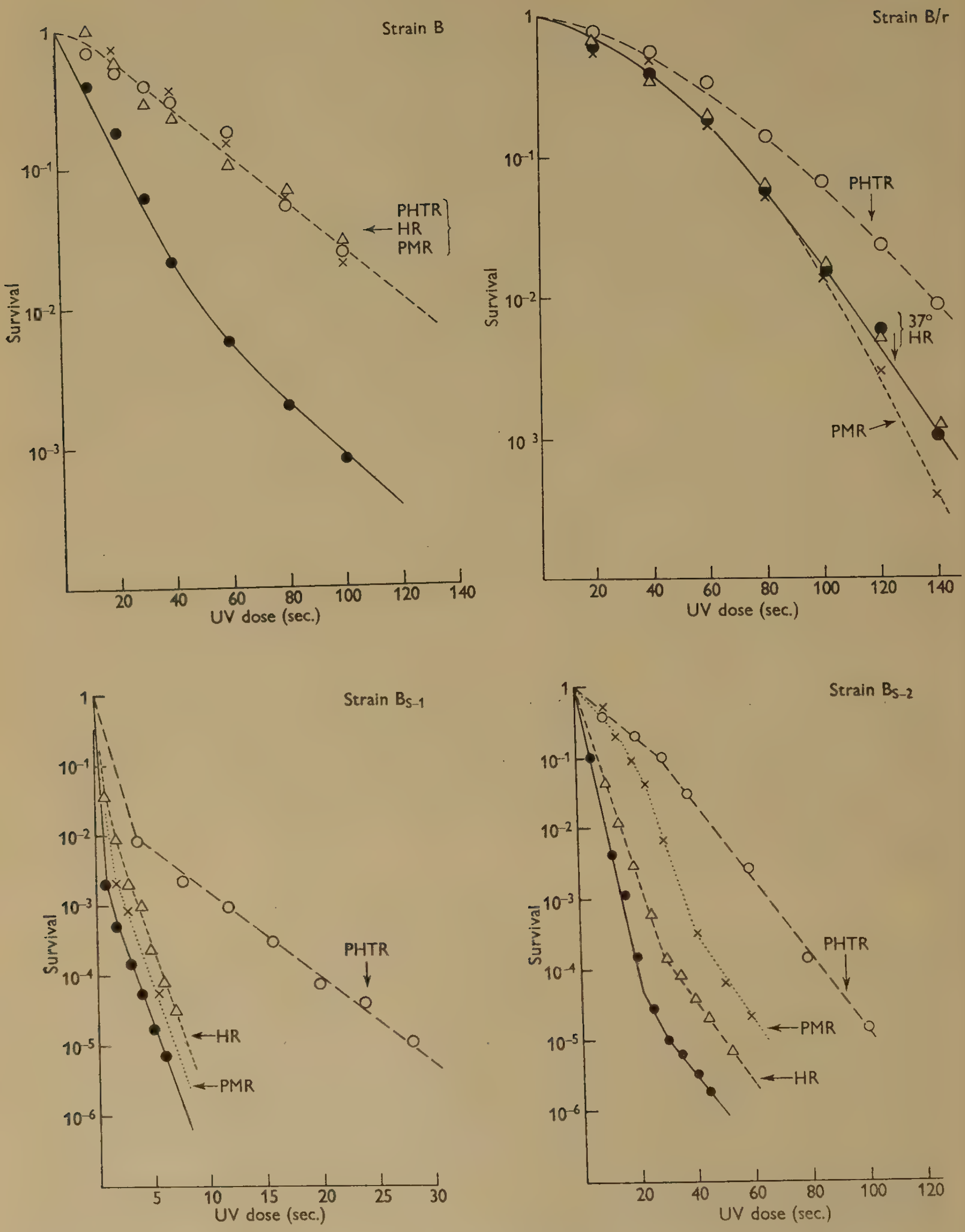

Fig. 2. Effect on survival of various treatments after u.v.-irradiation. PHTR = photoreactivation (illumination with wavelength 3650 Å); HR = heat reactivation (incubation at 45°); PMR = plating medium reactivation (incubation on M9 medium).

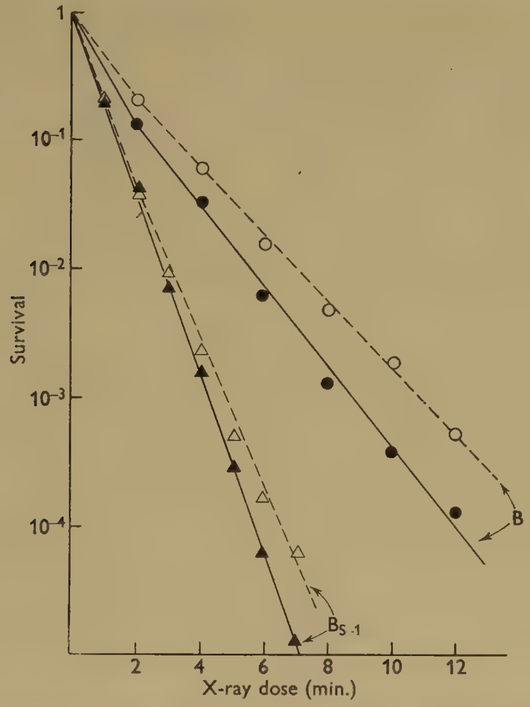

Fig. 3. Effect of incubation at 45° on survival of strains B and B_{s-1} after X-irradiation. ● and ▲, incubation at 37°; ○ and △, incubation at 45°.

Furacin sensitivity

The inhibitory concentrations of furacin (μg./ml.) were as follows: B/r—2·3, B—0·25, B_{s-1}—0·23 and B_{s-2}—0·53. As expected, B/r was markedly more resistant than the other strains. The value for B was the concentration inhibitory for a majority of B cells; the gradient pattern for B did not show a sharp break, but at concentrations greater than 0·25 μg./ml. the density of bacterial growth decreased (Payne *et al.* 1956). The gradient patterns for B_{s-1} and B_{s-2} showed sharp breaks at the above furacin concentrations. It was concluded that the furacin sensitivity of B_{s-1} was the same as that of a majority of B cells, while that of B_{s-2} corresponded to that of a minority of B cells.

Nuclear multiplicity, DNA and RNA contents

No significant differences between the four strains were observed with respect to these properties (Table 1).

In addition to these studies, the DNA of strains B, B_{s-1} and B/r was extracted by S.M. Beiser of the Department of Microbiology, Columbia University. Chromatographic fractionation on Ecteola-cellulose columns was performed by A. Bendich of the Sloan-Kettering Institute (Bendich, Pahl & Beiser, 1956). No obvious differences in profile between the strains were observed.

Table 1. *Nuclear multiplicity, DNA and RNA contents*

Strain	Average nuclear no.* of cells growing on agar for		DNA (μg./cell)	RNA (μg./cell)
	15 min.	2 hr.		
B_{S-1}	1·23	2·00	$6{\cdot}5 \times 10^{-9}$	$3{\cdot}3 \times 10^{-8}$
B_{S-2}	1·20	1·92	$6{\cdot}3 \times 10^{-9}$	$3{\cdot}2 \times 10^{-8}$
B	1·27	1·85	$7{\cdot}5 \times 10^{-9}$	$4{\cdot}2 \times 10^{-8}$
B/r	1·25	1·83	$6{\cdot}2 \times 10^{-9}$	$2{\cdot}9 \times 10^{-8}$

* These numbers are the averages of values obtained by three observers, each of whom counted 200 cells per strain.

Spontaneous mutation

The spontaneous mutation rate to T_1-resistance was essentially the same for the four strains (Table 2). On the other hand, mutation to the B_S forms produced a striking change in the subsequent ability to mutate to furacin-resistance (Table 3). The parent strain B mutated to furacin resistance at the rate of 10^{-5}/bacteria/generation, in agreement with the reported value for the rate of mutation to radio-resistance (Witkin, 1947). However, in a total of approximately 4×10^{11} plated bacteria of strain B_{S-1}, no furacin-resistant mutants were detected. It seemed possible that such mutants might have been produced but unable to survive under conditions of growth in the presence of the parent furacin-sensitive B_{S-1} bacteria. This was tested by growing mixtures containing known initial percentages of B_{S-1} and B/r bacteria

Table 2. *Spontaneous mutation to T_1-resistance*

Vol. of each culture = 10 ml. Vol. of samples = 0·1 ml.

Culture no.	Strain			
	Numbers of mutants in culture			
	B_{S-1}	B_{S-2}	B	B/r
1	169	26	170	319
2	61	123	77	29
3	57	102	22	564
4	165	47	24	38
5	66	28	80	17
6	34	3	4	43
7	153	31	18	38
8	79	32	32	37
9	29	19	28	101
10	72	18	78	197
11	33	61	18	23
12	15	65	42	39
13	98	84	18	54
14	23	194	182	14
15	82	431	36	72
16	100	297	21	15
17	88	149	24	112
18	35	100	55	13
19	415	64	29	46
20	29	30	33	11
Aver. no. mutants/culture	9000	9500	4950	8910
Bacteria/culture	$1{\cdot}9 \times 10^{10}$	$1{\cdot}9 \times 10^{10}$	$9{\cdot}6 \times 10^{9}$	$1{\cdot}4 \times 10^{10}$
Mutation rate	$4{\cdot}8 \times 10^{-8}$	$5{\cdot}2 \times 10^{-8}$	$5{\cdot}5 \times 10^{-8}$	$6{\cdot}4 \times 10^{-8}$

Table 3. *Spontaneous mutation to furacin resistance*

Method 1: cultures grown without aeration; samples spread on surface of agar containing furacin.

Method 2: cultures grown with aeration; samples added to melted agar containing furacin; agar poured into Petri dish.

	Strain				
	B_{S-1}		B_{S-2}	B	
Method	1	2	1	1	2
Vol. culture (ml.) ...	3	10	3	3	10
Vol. sample (ml.) ...	0·01	1	0·01	0·01	0·001
	No. of mutants in culture				
Culture no.					
1	0	0	0	554	35
2	0	0	0	16	15
3	0	0	0	11	30
4	0	0	1	10	41
5	0	0	0	432	36
6	0	0	0	9	6
7	0	0	0	7	90
8	0	0	1	46	17
9	0	0	0	374	23
10	0	0	2	2	32
11	0	0	0	6	19
12	0	0	0	4	29
13	0	0	3	947	3000
14	0	0	0	17	30
15	0	0	1	7	12
16	0	0	0	17	21
17	0	0	0	8	24
18	0	0	0	9	30
19	0	0	0	40	14
20	0	0	3	16	13
Aver. no. mutants/culture	0	0	0·55	$3{\cdot}8 \times 10^{4}$	$1{\cdot}8 \times 10^{6}$
Bacteria/culture	$7{\cdot}32 \times 10^{8}$	$1{\cdot}9 \times 10^{10}$	$4{\cdot}8 \times 10^{8}$	$3{\cdot}8 \times 10^{8}$	$6{\cdot}9 \times 10^{9}$
Mutation rate	0?	0?	$5{\cdot}4 \times 10^{-8}$	$9{\cdot}0 \times 10^{-6}$	$1{\cdot}25 \times 10^{-5}$

and then determining the final composition. The results showed that instead of failing to compete successfully with B_{S-1} bacteria, the final percentages of B/r were somewhat higher than expected (Table 4). Therefore it appears that either B_{S-1} mutates to furacin resistance, i.e. to the B/r form at a greatly reduced rate compared to the rate for strain B, or does not mutate at all.

Table 4. *Survival of strain B/r when grown with strain* B_{S-1}

The strains were grown separately in nutrient broth, diluted and then mixed in various ratios. The total bacterial concentration in each mixture was $9{\cdot}2 \times 10^{4}$ per ml. The mixtures were grown to saturation and tested for the percentage of each component by diluting and plating on nutrient agar with and without 1·5 μg. furacin/ml.

Initial % B/r	50	10	1·0	0·10
Final total titre	$3{\cdot}0 \times 10^{8}$	$2{\cdot}8 \times 10^{8}$	$3{\cdot}1 \times 10^{8}$	$2{\cdot}1 \times 10^{8}$
Final titre as determined by plating on agar containing furacin	$2{\cdot}6 \times 10^{8}$	$7{\cdot}3 \times 10^{7}$	$8{\cdot}6 \times 10^{6}$	$3{\cdot}5 \times 10^{7}$
Final % B/r	86	26	2·8	0·17

In the case of B_{s-2}, eleven furacin-resistant colonies appeared in a total of 10^8 bacteria plated. This gave a mutation rate of $5 \cdot 4 \times 10^{-8}$—considerably less than that for strain B. These eleven colonies were tested for (*a*) sensitivity to phage T_1 in order to eliminate possible contaminants; (*b*) cytological response to u.v. irradiation; (*c*) survival to a test dose of u.v. radiation; (*d*) inhibitory concentration of furacin. No contaminants were found. According to criteria (*b*), (*c*) and (*d*), one colony consisted of typical B/r bacteria. However, the remaining ten colonies (not all independent) were peculiar. Their cytological response to u.v. was not entirely characteristic of B_{s-2}, since many, instead of a few, short filaments, were formed after 3 hr. incubation and in addition they showed confluent growth after 24 hr. Furthermore, these colonies resembled B/r in furacin resistance, but their survival to the test dose of u.v. was like that of B (Table 5).

Table 5. *Properties of colonies originating from strain B_{s-2} plated in the presence of furacin*

	% surv. to test dose of u.v.	Snake formation	Inhibiting concn. furacin (μg/ml.)
Strain B control	1·9	+	0·25
Strain B/r	77	0	2·8
Colony no.			
1	1·2	0	2·4
2	1·5	0	2·3
3	1·7	0	2·3
4	17*	0	>5
5	0·91	0	2·6
6	1·1	0	2·6
7	2·4	0	2·5
8	1·2	0	2·6
9	0·90	0	3·0
10	0·64	0	3·0
11	0·95	0	3·3

* Although this % survival is lower than the value for the particular strain of B/r used in this experiment, it agrees with values for three other strains of B/r in our laboratory.

DISCUSSION

Several investigators have reported that strains of B/r characterized generally by increased radioresistance (compared to that of the parent strain B) constitute a heterogeneous group when examined more critically (Witkin, 1947; Kohn & Gunter, 1956; Adler & Haskins, 1960). Although only two mutants showing increased radiosensitivity were studied here, these also were found to be different and distinguishable by their response to u.v.-irradiation and to ^{32}P decay. The coincidence of the survival curves for X-irradiation may have been due to the choice of experimental conditions. Strain B_{s-1} could be distinguished from B_{s-2} since the survival after X-irradiation of the former could be increased by incubating for colony formation at an elevated temperature.

The present results show that neither the nuclear multiplicity nor the nucleic-acid content are responsible for the differences in sensitivity between strain B and the two types of radiological mutants, Br and B_s. Similar conclusions regarding

B and B/r have also been drawn by Gillies & Alper (1960). Evidently these differences are due to changes at the gene level. The present failure to find corresponding differences in the chromatographic profiles of the DNA of these strains is disappointing but probably due to an inadequate resolving power (A. Bendich, personal communication).

Since the function of the gene is to determine metabolic processes in the cell, changes in radiosensitivity reflect changes in these processes. For a given strain, the radiosensitivity may change if experimental conditions are varied, for example, by the application of different post-irradiation or post-decay treatments. The present results show that PHTR, HR and PMR were all effective in increasing the survival of B, B_{s-1} and B_{s-2} after u.v.-irradiation, although the relative effectiveness of these three kinds of reactivation was different for each strain. However, only PHTR was effective for strain B/r. After X-irradiation and after ^{32}P decay, HR was effective for B and B_{s-1} but not for B_{s-2} and B/*r*. This failure to increase the survival of B/r by post-treatments (other than PHTR) is also evident in the work of Alper & Gillies (1960) in which it was shown that B and B/r were equally radiosensitive under certain conditions. This equality resulted from a decreased resistance for B/r and an increased resistance for B when the strains were u.v.-irradiated during the rapid growth phase and then plated on defined medium instead of the usual broth. Similarly, Okagaki showed that post-u.v. treatment with chloramphenicol increased the resistance of B but was lethal for B/r (1960). Thus although the mutation of B to the B_s form confers an obvious disadvantage as far as inactivation is concerned, it is interesting that mutation to the B/r form can be disadvantageous with respect to reactivation.

The finding that the four strains did not differ in their spontaneous mutation rates to T_1-resistance can be interpreted as further evidence that the genetic differences between them do not involve the *whole* genetic apparatus but only a limited part, probably one or a few genes. This is corroborated by the very different rates of mutation of B, B_{s-1} and B_{s-2} to furacin resistance, i.e. mutation at a locus which is related to increased radioresistance.

Since, as mentioned above, strains of B/r have been found to be heterogeneous, it seems possible that strain B contains a number of loci such that mutation at any one confers increased radio and furacin resistance. Only two studies to date have been reported involving recombination between radioresistant strains. Bryson & Tuttle (private communication) crossed *Escherichia coli* B/r and *E. coli* K12, which is also radioresistant as compared to B. Some of the recombinants were radiosensitive in that they were snake formers like B. However, this could have resulted either from the presence of a single radioresistance gene in non-identical positions in B/r and K12, which are unrelated strains or from the presence of multiple loci in either or both strains. More recently, Zelle & Ogg reported recombination data involving three strains of B/r, one of which was characterized by increased cell size and increased radioresistance as compared to the other two B/r types (1957). The large cell variant is believed to be diploid or polyploid so that recombination might involve whole chromosomes. Thus the data concerning this strain do not illuminate the question of the possible multiplicity of radioresistance loci in the usual, presumably haploid, B/r. Unfortunately, the presence of the B phenotype was not looked for among the recombinants of the other two B/r strains.

In the absence of this kind of data, it can be assumed at present that strain B does contain a number of 'radioresistance' genes. Since the present findings also indicate heterogeneity of B_s types, it might be also assumed that strain B contains a number of 'radiosensitive' genes. The evidence with respect to B_{s-2} suggests that there is at least one such gene. In particular the finding of B-like forms in irradiated populations of B_{s-2} indicates that reversion at this presumed locus can occur. If B_{s-2} were to mutate at one of the radioresistance loci, the resulting strain might have just the hybrid properties found for the ten colonies on furacin plates, i.e. such strains might be furacin-resistant like B/r, but since there has been mutation of two genes, one of which confers *increased* and the other *decreased* radioresistance, the resultant radiosensitivity might be something in between that of B_{s-2} and that of B/*r*, namely, that of B.

Strain B_{s-2} also yielded one mutant which was typically B/r—both furacin- and radioresistant. This could have arisen in at least one of two ways. The extemely low frequency of this B/r—one in a total of 10^{10} bacteria plated—suggests that there may have been two mutations in B_{s-2}. These might both have occurred at radioresistance loci. The second such mutation might raise the radioresistance from that found for the putative hybrid strains to a level much more like that of a typical B/r. Alternately, this B/r mutation might have arisen from a reversion at the B_{s-2} locus itself followed by a mutation of the resulting B-type cell to B/r. It was mentioned that in one culture of B_{s-2}, 85 % of the colonies obtained after a large dose of u.v. were like strain B (snake formers) whereas in another culture, only 1 % such colonies were found. This variation in frequencies suggests that the B-like forms were surviving spontaneous revertants. If so, then the mutation of B_{s-2} to B would have to occur spontaneously at a fairly high rate. Since the mutation rate of B to B/r is of the order of 10^{-5} (Witkin, 1947), an over-all frequency of 10^{-10} for the mutation B_{s-2} to B to B/r does not seem unreasonable. A choice between these two possible modes of origin of B/r from B_{s-2} must await attempts to detect selectively B in B_{s-2} populations and consequently to measure the mutation rate of B_{s-2} to B.

The findings with respect to B_{s-1} do not quite fit in with the idea that B contains a number of radiosensitive loci. In this case, neither furacin-resistant mutants nor B-like forms were detected. The simplest explanation is that the radiological loci are missing in this strain, i.e. that it results from a deletion in strain B. The recent report that u.v.-irradiated phages T_1, T_3 and T_7 show a considerable decrease in plaque count with B_{s-1} compared with B and B_{s-2} is in agreement with this hypothesis (Ellison, Feiner & Hill, 1960). It might be supposed that the chromosomes of B, B_{s-1} and B_{s-2} contain loci necessary for the replication of normal phages of these three types but that the chromosome of B_{s-1} alone is missing a piece necessary for the growth of u.v.-damaged phage. This piece might also contain the radiological loci.

A last point to be considered is the finding that the mutation of B to B/r was accompanied by increased furacin resistance, whereas mutation to either of the B_s forms did not increase furacin sensitivity. Apparently only the radioresistance genes can alter furacin sensitivity. This suggests that both properties result from the same alteration in cellular metabolism. Therefore it would be interesting to determine whether, under conditions in which the radioresistance of B/r is markedly decreased, its furacin resistance is decreased also and conversely whether, when the radioresistance of B is increased, there is an increase in its furacin resistance. One possible set of conditions is that described by Alper & Gillies (1960).

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The Measurement of Bacterial Viabilities by Slide Culture

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SUMMARY

A procedure is described for measuring the viabilities (% viable/total organisms) of populations of *Aerobacter aerogenes* by short-term incubation on agar films followed by differential counting under the microscope. The only special apparatus needed is a supply of metal annuli. The procedure estimates viability with 95% fiducial limits of ±9%; it appears applicable to other aerobic bacteria, to yeasts and to spore suspensions.

INTRODUCTION

The viability of a microbial population may be defined pragmatically as the proportion of its members that are capable of multiplication when provided with optimal conditions for growth. In this usage an organism may be called dead if it does not multiply in those conditions, and certain philosophical difficulties in the concept of death as applied to micro-organisms (see, for example, Valentine & Bradfield, 1954; Powell, 1956) may be avoided. Many procedures are available for determining the viability in this sense, the most popular being that of obtaining simultaneous total and viable counts on the population. Other procedures include 'vital staining', based on the assumption that death is accompanied by an altered permeability to dyes, which has been used with yeasts (e.g. Townsend & Lindegren, 1953) and bacteria (e.g. Knaysi, 1935; Knaysi & Ford, 1938; White, 1947). Strugger (1948) modified this technique by using a fluorescent dye with preparations of soil bacteria and Razumovskaya & Osipova (1958) applied Strugger's method to pure cultures of *Acetobacter* spp. Techniques based on the belief that death is associated with inability to reduce a redox dye such as a tetrazolium have been reported (e.g. Greenberg, Eidus & Diena, 1958). Koch (1959) assumed that leakage of labelled purines from populations of labelled bacteria measured the extent of death in such populations. Barer, Ross & Tkaczyk (1953) proposed that immersion refractometry should distinguish dead from living cells since their refractive indices were often different; this technique was used by Firkhman (1959). Mager, Kuczynski, Schatzberg & Avi-dor (1956) showed that populations of live Gram-negative bacteria had a greater optical density in solutions than in distilled water, but that dead ones did not, and suggested the exploitation of this 'optical effect' for viability determination. Wade & Morgan (1954) developed a staining procedure which made use of the accumulation of ribonucleic acid that takes place before a viable organism divides; Valentine & Bradfield (1954) measured viability by incubating populations in 3% urea, which permitted growth but not division, and determined the ratio of giant to ordinary cells with an electron microscope. Powell (1956) grew bacteria on a cellophan grid and determined the ratio of the individual count before incubation to the group count after incubation; Taubeneck (1957, 1959) proposed modifications of Powell's procedure for slide cultures.

A critique of the methods available is not appropriate to the present paper, but their existence and the fact that none is widely accepted as both satisfactory and convenient perhaps justifies the publication of yet another one. The procedure reported below is a modification of the slide culture used by Knöll (1944) and others. The cellophan or Formvar grids needed for Powell's procedure or Taubeneck's modification are dispensed with; the method requires no special apparatus beyond microscope slides, metal annuli and a microscope set up for phase contrast or dark ground illumination. It has proved satisfactory as a routine procedure for studying the viability of microbial populations, provided these fall in the range 5–100 %.

METHODS

Preparation of agar cultures on slides. Brass annuli of 20 mm. internal diameter, 22 m. external diameter and 1 mm. depth were cut from piping; these support circular coverslips (nominally $\frac{7}{8}$ in., actually less) without overlap. They were cleaned, chemically blackened to facilitate observation of the sealing operation (below) and stored in 98 % (w/v) ethanol in water ready for use. Clean microscope slides 0·8–1 mm. thick were kept in ethanol, and before use both were burned dry and the annulus placed on the slide. A 0·22–0·24 ml. portion of hot agar medium (see below) was spread inside the annulus, thus sticking it to the glass, and the whole was stood under a Petri plate lid for 5 min. A portion of the population to be studied was diluted to *c* 100 million organisms/ml., spread on the surface of the agar film and allowed to stand for 10 min. If the surface of the agar was not dry by then, excess fluid was wiped off with a piece of clean filter paper.

A clean coverslip kept in ethanol was wiped with a cloth and placed on the annulus over the agar. The annulus-coverslip interface was touched with a loop of distilled water and capillarity caused water to flow between the two surfaces, thus sealing the agar under an air space. In spite of evaporation of the distilled water, the seal remained effective for many hours. The slide was incubated at a suitable temperature and for an appropriate period (37° and 2–4 hr. with populations of *Aerobacter aerogenes*) cooled and inspected under phase contrast either after cooling to room temperature or after storage for up to 18 hr. at 4°. Two stages in the preparation of these slides are illustrated in Pl. 1.

Preparation of agar. Commercial agar preparations and broths brewed from butchers' meat frequently contain solid debris and dead bacteria, both of which interfere with counts. We have usually found it desirable to filter agar media hot through a Millipore filter before use, though occasional batches have been obtained with which this expedient was not necessary. Blood agars were unsatisfactory because the corpuscles interfered with the phase contrast used for microscopic examination of the slides.

Inspection of slides. For bacteria and yeasts we have used a binocular microscope (magnification of ×360; field diameter: 0·29–0·32 mm.) with a 4 mm. objective arranged for phase contrast in green light. A condenser with a working distance sufficient to focus through the slide and agar film is necessary; we have successfully used microscopes supplied by Messrs Cooke, Troughton and Sims Ltd. and by

Messrs Watson Ltd. Mr R. E. Strange and his colleagues in this Establishment have used dark ground illumination satisfactorily. An objective having a long working distance is an advantage, since the slide may then be inspected without removing the coverslip, but fogging of the coverslip can be a nuisance and routinely we removed it for counts. Differential slides of pathogenic bacteria were sterilized by exposure to formaldehyde vapour before inspection.

Fields containing forty objects (dead bacteria or microcolonies) were usually chosen for counting, though with populations of low viability a larger number of objects per field was permissible. The microcolonies and dead organisms per field were counted separately. Sufficient whole fields, preferably adjacent, were counted to give a total of over 300 objects per slide studied. A tally counter or electric counting device is a great help in routine work of this kind. The viability was derived directly from the ratio of the number of microcolonies to the total number of microbial objects counted.

RESULTS

Aerobacter aerogenes (NCTC 417) obtained from Professor Sir Cyril Hinshelwood's laboratory was grown in continuous culture at 40° and at a dilution rate of 0·2–0·3 vol./hr. in a glycerol + salts medium resembling that used for *Cloaca cloacae* by Herbert *et al.* (1956) in which glycerol limited growth. Organisms were harvested in the logarithmic phase of growth, washed twice in a mixture of 0·137 M-NaCl (9 parts) + 0·048 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer (pH $7{\cdot}15 \pm 0{\cdot}15$; 1 part) and incubated in this buffer at 40° with gentle aeration to obtain moribund populations. In some experiments, for reasons unconnected with this paper, 0·316 mM ethylenediaminetetra-acetic acid (EDTA) was present. Data quoted refer to organisms harvested between the 250th and 1000th generations in continuous culture.

Growth on slide cultures

As a preliminary, some fresh or moribund populations were watched from shortly after inoculation until considerable multiplication of the viable bacteria had taken place. Records were kept of the behaviour of the individual organisms. The following points were established:

(i) When a large proportion of the bacteria were viable, microcolonies tended to over-grow the non-viable organisms. Hence fields containing more than 30–40 objects should be avoided.

(ii) In populations showing a high viability, all organisms that were going to divide had done so by the time the earliest starters had undergone two or three divisions. With moribund populations (less than 30 % viability) the scatter of individual division lags was wider and more prolonged incubation was desirable.

(iii) Moribund populations contained a small proportion of organisms that appeared to attempt growth but to fail. They 'died' as drumstick-shaped or spherical bodies of low contrast. Such organisms were encountered most often in populations of intermediate (70–20 %) viability.

(iv) Loss of organisms owing to lysis was rare (usually a recognizable 'ghost' could be seen) and restricted to populations of less than 20 % viability.

(v) Loss of organisms owing to lysis or overgrowth occurred most often when the average division lag was long. Hence media rich in nutrient supplements were desirable. In the case of *Aerobacter aerogenes* the medium of choice was that used for continuous culture of the organism supplemented with 0·1 % (w/v) Difco yeast extract, 0·1 % (w/v) Difco Casamino acids, 1/10th volume of home-brewed tryptic meat broth and Difco agar to 1·5 % (w/v). On this medium satisfactory differential counts were obtained after 2–4·5 hr. at 37°; moribund populations required the longer period of incubation.

Evaluation of the method

The reproducibility of the method with a population of bacteria dying 'naturally' was studied in the following manner. Organisms harvested from continuous culture at 40° were washed twice in saline + tris buffer, suspended in distilled water to their original concentration and 1 ml. of this suspension added to four 50 ml. portions of saline + tris buffer. The population was then *c.* 5×10^7 organisms/ml. The tubes were incubated at pH $7{\cdot}0 \pm 0{\cdot}2$ and 40° with gentle aeration; one tube (A) was sampled at fairly frequent intervals during 10 hr., single differential counts being performed on the population. On some of these occasions tube A was tested in quadruplicate and then single differential counts on the other three tubes were also performed, thus providing blocks of seven tests on populations that should have had identical viabilities. These blocks of seven tests were performed in closely spaced pairs intended to bracket a high, a medium and a low viability of the population.

The counts were performed by three operators, none of whom 'specialized' in counting samples from a given suspension; each had his own microscope. On one occasion (9 hr.) the operators re-counted each others' slides; Fig. 1 includes all the counts then obtained. The death curve of this population was essentially linear (Fig. 1); apparent tendencies to a sigmoid form suggested by inflexions during the first two and last hours fell within the 95 % fiducial limits of the regression line and the death curve has been drawn without them. (Other experiments on this population indicated that the later inflexion was probably real.) Some of the data from which Fig. 1 was plotted are given in Table 1. A χ^2 test on the actual counts from which these data were obtained showed excellent agreement within each set of seven counts; the usual 95 % fiducial limits of the regression line were ±9·2 %; the viabilities of the four suspensions did not tend to appear in any order during the 10 hr. of the experiment.

Comparison with a conventional viability determination. A second death curve was obtained by using ordinary plating-out procedures, combined with fourteen separate total counts by three operators on the initial population using two Thoma counting chambers of 0·03 mm. depth. This procedure gave a mean population of $5{\cdot}9 \times 10^7$/ml. but in our hands the standard deviation of the technique was $\pm 1{\cdot}44 \times 10^7$ (counts by individuals showed greater reproducibility). Hence the error of the absolute viability from this source was 25 % without taking account of errors in the plating-out procedures. The experiment is illustrated in Fig. 2. The death curve obtained by the conventional procedures resembled that obtained by the differential procedure, but the viabilities were systematically lower. This difference is most likely due to errors in the total count; when viabilities were expressed as percentages of the initial plate count (taken as 97 %, the value indicated by the differential count), the death curves followed very similar courses.

Table 2 illustrates some data obtained with a population which survived for a considerably longer period; there is close agreement between the differential count and the colony count, based on the assumption that the initial colony count represented *c.* 100% viability. These data were obtained in this laboratory by Mr R. Strange and his colleagues; as indicated in the legend, they refer to stationary phase *Aerobacter aerogenes* grown in conditions different from those used by ourselves.

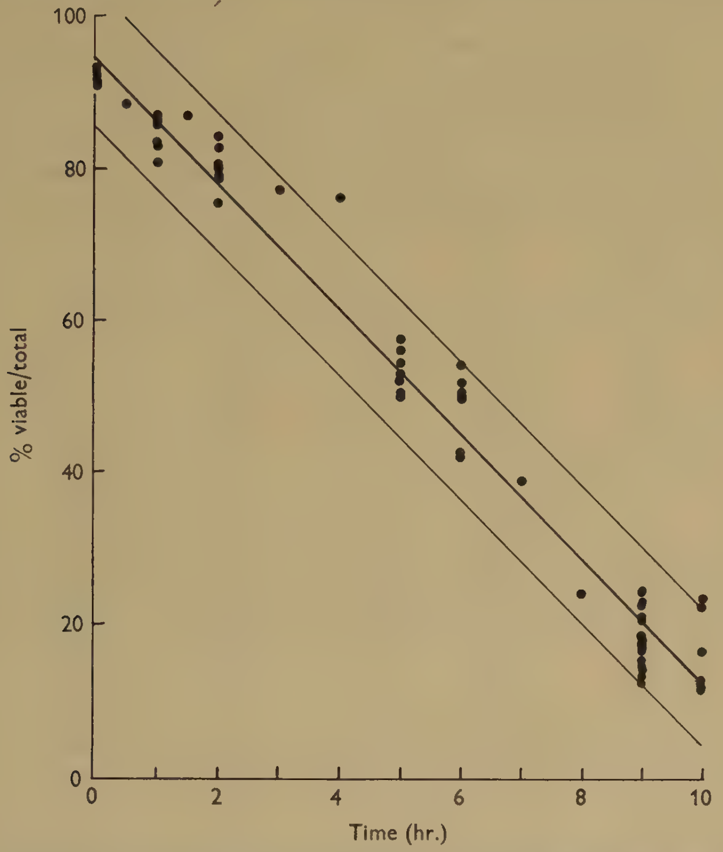

Fig. 1. Death curve of log phase *Aerobacter aerogenes* in aerated saline + tris buffer at 40°. Viabilities of four similar populations of *A. aerogenes* were determined by three operators using the differential counting procedure. For methods and sampling plan see text; for selected data see Table 1.

Various applications of the method

Use with bactericides. The procedure provides a rapid method of determining the proportion of a population killed by a bactericide and may be used to follow the killing rate of such a compound, provided the kill can be expressed in percentages rather than powers of ten. Table 3 shows titrations of the immediate bactericidal effects of methylene blue, aureomycin and proflavine on *Aerobacter aerogenes* harvested in the logarithmic phase; Fig. 3 shows a death curve in which methylene blue exerted a delayed bactericidal effect even though its concentration was insufficient to show an immediate effect.

Use with freeze-dried material. The procedure is useful for assessing the viability of suspensions before and after freeze-drying. A population of log phase *Aerobacter aerogenes* had a viability of 95·1 %; after freeze-drying its viability was 4·4 % by a combination of conventional total and viable counts; 4·1 % by the differential counting procedure.

Use with other micro-organisms. Several microbes have been grown on slides in the manner described to see whether their microcolonial appearance was suitable for use by the method. Those that were successful are listed, with the temperature of incubation and the incubation periods at which the differential count was clear and constant recorded in parentheses. *Pseudomonas ovalis* (30°; incubated for 2, 3½ hr.), *Serratia marcescens* (37°; 1½, 2 hr.), *Pasteurella pestis* (28°; 4½, 6 hr.), *Salmonella typhimurium* (37°; 2, 3 hr.), *Micrococcus* 'M9' (37°; 3½, 5 hr.), *Bacillus subtilis* (37°; 2, 3 hr.), *Alkaligenes metalcaligenes* (18–20°; 17, 19 hr.), bakers' yeast (18–20°; 18, 22 hr.). All bacteria were tested in home-brewed tryptic meat agar; that for *P. pestis* was supplemented with haematin. 'Viability' of the micrococcus was obtained as percentage viable units in the population because the strain showed many pairs and tetrads before incubation; hence the relatively long incubation periods. Bakers' yeast (pressed yeast from Distillers Co. Ltd.), which was grown on a medium containing malt extract, peptone, yeast extract and glucose, showed

Table 1. *Representative sample of experimental material from which Fig. 1 was obtained*

Viabilities of four similar dying populations of *Aerobacter aerogenes* were determined by three operators using the differential counting precedure. For methods and sampling plan, see text.

Time of incubation (hr.)	Population tested (A, B, C or D)	Micro-colonies	Dead organisms	Viability (%)	Person counting	Time slide culture was incubated (hr.)
0	A_1	292	26	91·8	JRP	2
0	A_2	280	21	93	JRH	2
0	A_3	289	21	93·2	JRH	2
0	A_4	282	20	93·4	JRH	2
0	B	285	29	90·8	JEC	2
0	C	285	21	93·1	JEC	2
0	D	301	24	92·6	JRP	2
0·5	A	273	33	89·2	JRH	2
1	A_1	300	44	87·2	JRH	2·5
1	A_2	260	51	83·6	JRP	2·5
1	A_3	261	52	83·4	JRH	2·5
1	A_4	269	43	86·2	JRH	2·5
1	B	247	57	81·2	JEC	2·5
1	C	279	44	86·4	JRP	2·5
1	D	292	40	86·6	JRH	2·5
1·5	A	274	40	87·2	JRP	2·5
6	A_1	166	153	52	JRP	2·5
6	A_2	144	196	42·3	JRH	2·5
6	A_3	156	150	50·9	JEC	2·5
6	A_4	152	148	50·6	JEC	2·5
6	B	144	194	42·6	JRH	2·5
6	C	158	158	50	JRP	2·5
6	D	172	143	54·6	JRP	2·5
7	A	120	187	39·1	JEC	2·75
8	A	75	236	24·1	JEC	2·7

91% viability on slide culture and 90% viability by the methylene blue vital staining procedure (Townsend & Lindegren, 1953) when fresh; after 14 days in buffer at pH 7 ± 0·3 and room temperature the slide procedure gave a viability of 62% compared with 18·5% indicated by the methylene blue procedure. The procedure provided a rapid spore germination test with suspensions of *B. subtilis*, *B. megaterium* and *B. cereus* spores.

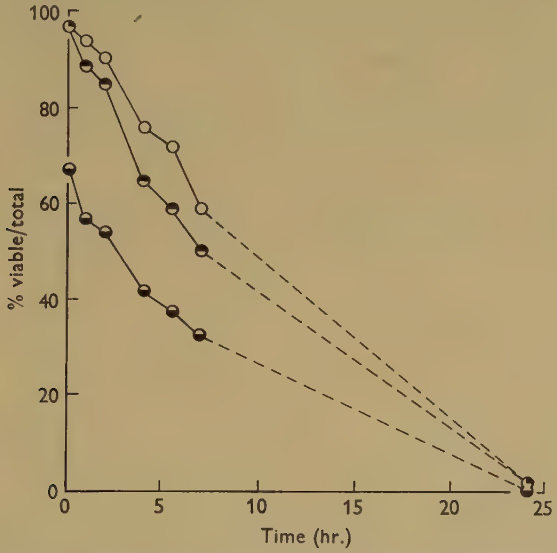

Fig. 2. Death curves of a population of log phase *Aerobacter aerogenes* obtained by conventional procedures and by slide culture. Logarithmic phase *A. aerogenes* were washed by centrifugation and incubated in saline + tris buffer containing a small amount of EDTA at 40°, pH 6·8 ± 0·05. Samples were removed at intervals for viability determinations (see text). ◒ Conventional procedure; ○ differential procedure; ◓ colony counts as % initial colony count.

Table 2. *Comparison between colony and differential counts on a population of* Aerobacter aerogenes

A. aerogenes grown in tryptic meat broth and harvested after the population density had become stationary for 0·5 hr. were incubated with aeration at 37° in NaCl (0·13 M) + KH_2PO_4/K_2HPO_4 (0·02 M), pH 6·50 ± 0·05.

Incubation period (hr.)	0	16	24	40	48	64	72	88
Colony count/10^{-8} ml.	98	95	90	96	95	75	73	60
Differential count (%)	97·5	98	96	90	87	78	68	59

Organisms for which the method was not suitable were *Escherichia coli* type B (37°; 1¼–3½ hr.) and *Bacillus megaterium* (*c.* 37°, *c.* 2½ hr.), both of which formed a mixture of ordinary cells and long ramifying filaments which obscured the slide.

DISCUSSION

The determination of viability directly by slide culture has certain obvious limitations. The method as described is limited to non-filamentous aerobes; bacteria with division lags longer than the incubation period are counted as dead; all organisms that have divided once are counted as live, even though both may occasionally be dead. The method is at its most exact at 50% viability, and is highly

Table 3. *Bactericidal effects of three inhibitors on* Aerobacter aerogenes

Bacteria were harvested from continuous culture at 40°, washed twice and populations of *c.* 6×10^7 organisms/ml. exposed to bactericide solutions in saline + tris buffer (pH 7), centrifuged and re-suspended in saline + tris buffer. The viable populations were then determined by slide culture. Exposure to inhibitor and centrifugation took about 10 min. at room temperature. Controls without dye were also centrifuged.

Bactericide	μg./ml.	Viability (%)
Methylene blue	—	90·7
	5	82·3
	10	55·9
	20	39·1
	40	7·6
	80	1·3
Proflavine	—	92·9
	10	87·4
	20	89·3
	40	82·7
	80	62·4
Aureomycin	—	96·5
	10	91·7
	20	94·7
	40	86·7
	80	56·1
	100	39·3

insensitive at viabilities below, say, 5 %; errors due to overgrowth of dead organisms by microcolonies or to lysis on the agar film exist and may assume importance with organisms other than *Aerobacter aerogenes*. Moribund cultures often yield morphological aberrants and experience is needed to decide whether the organism is living,

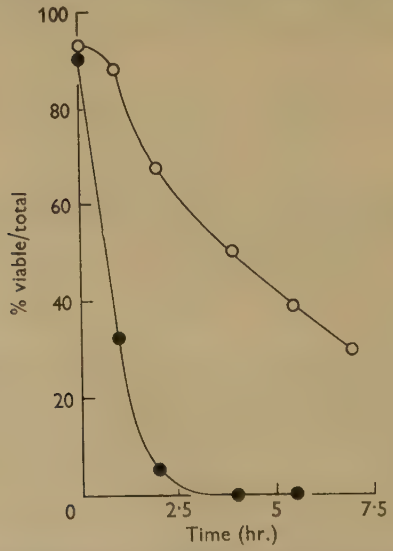

Fig. 3. Progress of bactericidal action of methylene blue on log phase *Aerobacter aerogenes*. Suspensions of *c.* 5×10^7 washed organisms/ml. saline + tris buffer containing EDTA aerated at pH 7·05 ± 0·05 and 40°. 1 ml. samples were removed at intervals, washed once by centrifuging to remove inhibitor and the viabilities determined by slide culture. ○ Control; ● methylene blue, 5 μg./ml.

dead, or a contaminant. The method has no obvious use in the counting of anaerobes; it cannot be used with populations totalling less than *c.* 10^7 organisms/ml. Within these limitations, however, the method has one or two advantages. The operator has added confidence in the method because he actually sees the viable and non-viable organisms instead of deducing their presence from altered staining properties, permeability, dehydrogenase activity or refractive index. The ordinary determination of the ratio of total to colony counts is slow as well as being beset by two sets of experimental errors; it is probable that the differential count described here gives a considerably more accurate ratio provided the two counts fall within a tenfold range of each other. Finally, though it is probably inferior in accuracy to other methods based on direct observation of growth of individual organisms (Valentine & Bradfield, 1954; Powell, 1956), it requires considerably less specialized apparatus.

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EXPLANATION OF PLATES

PLATE 1

Slide cultures for differential counts in preparation. The right-hand annulus contains agar but has not been covered. The left-hand annulus has been covered with a coverslip and is in process of being sealed with water from a loop.

PLATE 2

Photomicrograph of the surface of a slide culture of a moribund population of *Aerobacter aerogenes*. The population from which this slide was prepared had been harvested in the logarithmic phase of growth, washed by centrifugation and aerated for 1 hr. in saline + tris buffer with Versene at 40°, pH $7{\cdot}05 \pm 0{\cdot}05$. A portion was then incubated as a slide culture for 3·5 hr. Viability: 68 %. Phase contrast; magnification × 800.

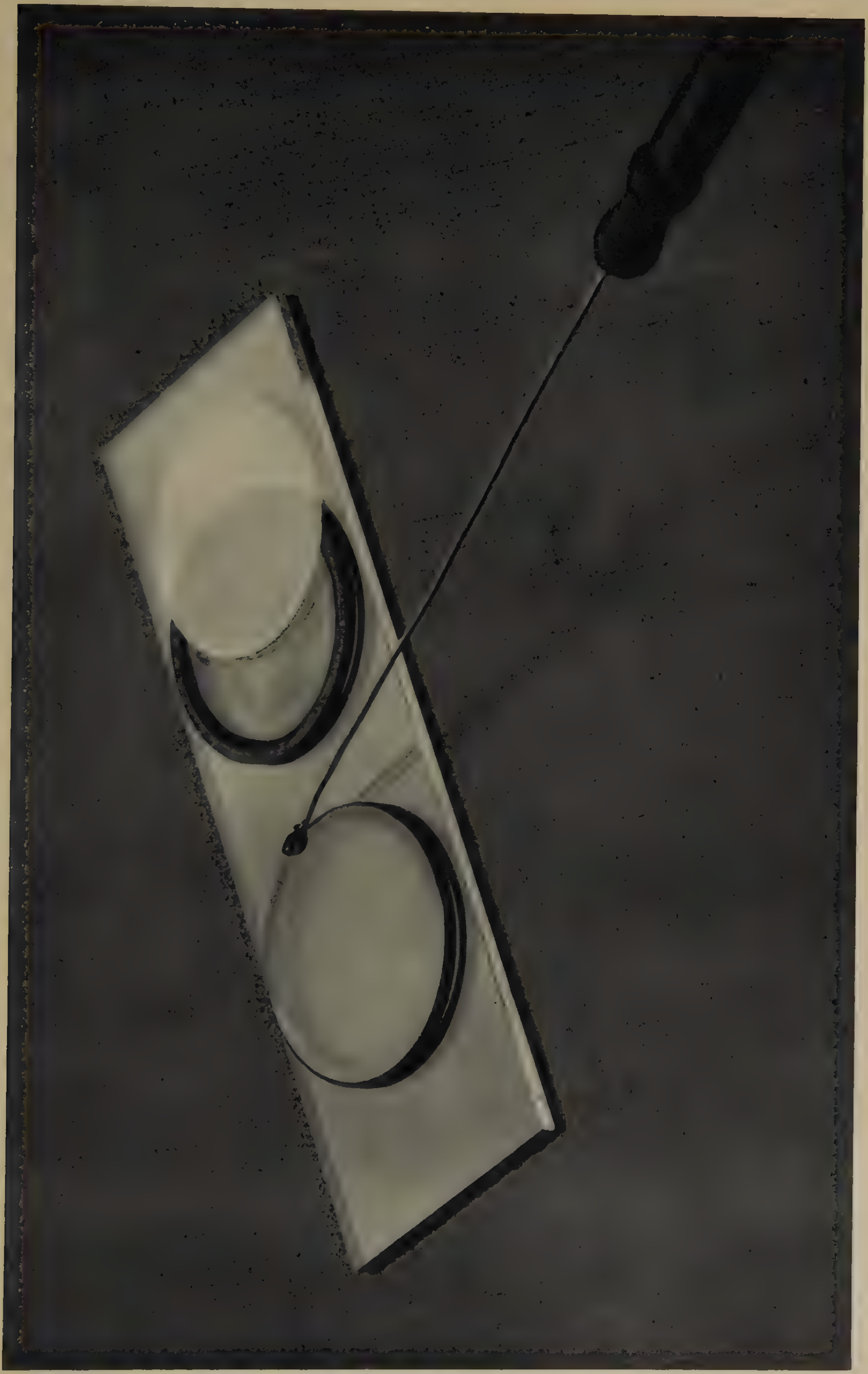
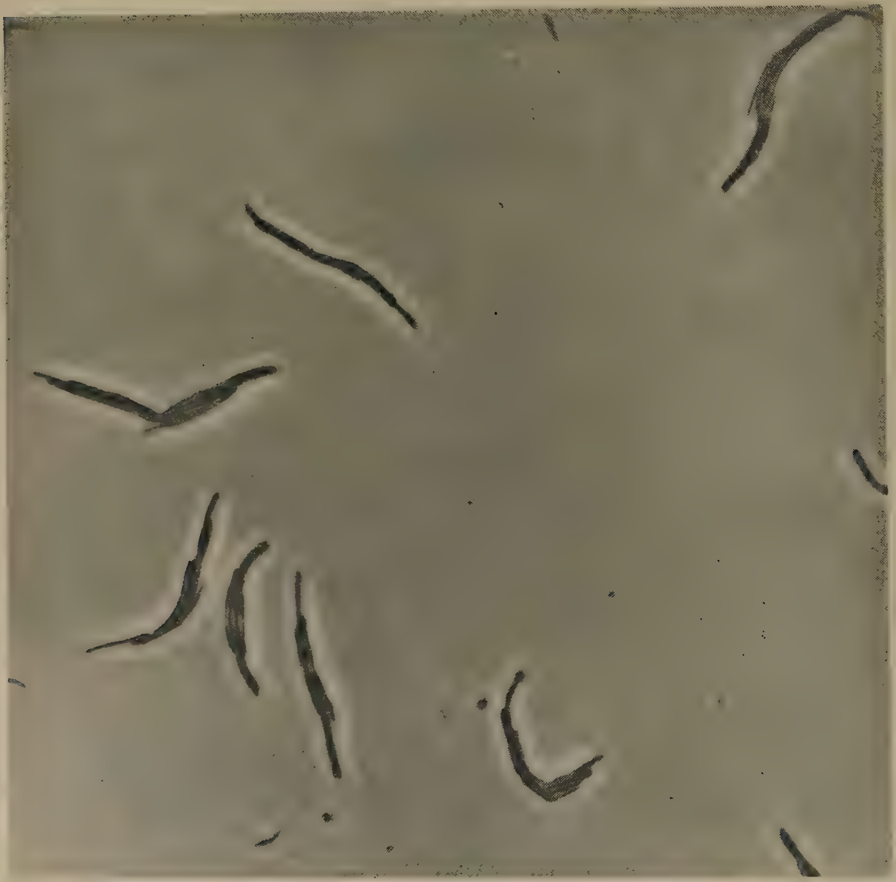


Plate 1

J. R. POSTGATE, J. E. CRUMPTON AND J. R. HUNTER

(*Facing p.* 24)



J. R. POSTGATE, J. E. CRUMPTON AND J. R. HUNTER

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The Effect of Asparagine on the Growth of a Gram-Positive Coccus

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(*Received* 31 *May* 1960)

SUMMARY

A *Pediococcus* sp., unusual in that it needed reducing conditions for growth, would not grow in a medium based on acid-hydrolysed casein but grew when asparagine was added. Neither glutamine nor aspartic acid + an ammonium salt satisfied this requirement. When the acid-hydrolysed casein was replaced by a mixture of amino acids, the omission of asparagine caused a decrease in growth in the presence of aspartic acid + an ammonium salt. No growth occurred in the absence of both aspartic acid and asparagine.

INTRODUCTION

Strains of Gram-positive cocci morphologically similar to *Pediococcus cerevisiae* but differing in other respects were described by Gunther (1958). Since a requirement for folinic acid may be a useful character for recognizing the pediococci (Felton & Niven, 1953), an attempt was made to determine this requirement for some of Gunther's strains. This was done in a casein hydrolysate medium based on that described by Sauberlich & Baumann (1948) for the assay of folinic acid with *P. cerevisiae* ATCC 8081. However, none of these strains grew in this medium even when folinic acid was present, although a normal response to this growth factor was given by the assay organism. It was found that growth occurred when Tween 80 and tryptic digest of casein were added to the medium (Garvie & Gregory, to be published). Therefore, a search to identify the active component in the tryptic digest was undertaken.

METHODS

Test organism and its maintenance. Strain NCDO 1250, sent by Dr H. L. I. Gunther under the reference number S344, was used. It is a catalase-negative Gram-positive coccus, growing in pairs, tetrads and clusters but not in chains, and is considered to belong to the genus *Pediococcus*. It differs from other known strains of this genus in being unable to grow aerobically in normal media. It was grown anaerobically in a McIntosh & Fildes jar filled with 90 % (v/v) H_2 + 10 % (v/v) CO_2 or alternatively in the presence of air by adding 0·5 ml. of a 1 % (w/v) Seitz-filtered solution of cysteine hydrochloride or ascorbic acid to each 10 ml. of medium immediately before inoculation.

The strain was maintained in litmus milk supplemented with 0·3 % (w/v) Yeastrel, 1 % (w/v) glucose, 0·1 % (v/v) Tween 80, 1 % (w/v) chalk; cysteine hydrochloride was added aseptically. After incubation at 30° for 24 hr. the cultures were stored at 4°.

Preparation of inoculum. The strain was grown in tomato glucose broth medium (Briggs, 1953) modified by Dr M. E. Sharpe, and having the following composition: (%, w/v) 1·5, peptone (Oxoid); 2·0, glucose; 0·5, Yeastrel; 0·5, sodium acetate; 0·5, KH_2PO_4; 0·2, di-ammonium citrate; (%, v/v) 10·0, tomato juice (Oxoid); 0·1, Tween 80; 0·5, salts B (Snell & Strong, 1939); adjusted to pH 6·5 and autoclaved (15 min. at 10 lb./sq.in.). Cysteine hydrochloride was added aseptically as described above. Serial transfers with an inoculum of *c.* 0·01 ml. were made after incubation for 48 hr. at 30°. A 24 hr. culture of the 3rd–5th transfer was used as the inoculum for the growth tests; one drop of a 1/100 dilution in quarter strength Ringer solution was inoculated into each assay tube.

Growth tests. The basal media (Table 1), which were modifications of that of Sauberlich & Baumann (1948), were made up at double strength and added in 5 ml. quantities to optically matched test tubes containing 5 ml. of test solution. The tubes were then covered with aluminium caps and autoclaved (10 min. at 10 lb./sq. in.). They were inoculated with strain NCDO 1250 and incubated at 30° for 48 hr., either aerobically or anaerobically, as described above. Relative amounts of growth were estimated turbidimetrically with a Lumetron 400-A colorimeter (Photovolt Co., New York).

Amino acid requirement of strain NCDO 1250. The growth of the cultures was measured in the defined medium A from which the amino acids were omitted, each in turn. Since cystine was one of the amino acids under test cysteine could not be used as a reducing agent and ascorbic acid was used instead in the tests incubated in air. Cultures which grew in the first test were serially transferred in the same medium to eliminate carry over of nutrients.

Estimation of the amino acid content of acid-hydrolysed casein and Tryptone by paper chromatography. Aqueous solutions (1 %, w/v) were adjusted to pH 2 by adding N-HCl and desalted on Dowex 50 (H^+) resin columns. The desalted eluates were concentrated under reduced pressure at 50° and used to prepare two-dimensional chromatograms on no. 1 Whatman paper by the procedure of Levy & Chung (1953).

Chemical estimation of glutamine and asparagine in Tryptone. Five ml. of 1 % (w/v) aqueous Tryptone (Oxoid) was hydrolysed in N-HCl for the estimation of total amide-N, and in borate phosphate buffer at pH 6·5 for the estimation of the amide-N of glutamine (Vickery *et al.* 1935). The ammonia produced was distilled into boric acid containing a mixed indicator of bromcresol green and methyl red (Conway, 1957). Solutions of glutamine and asparagine were analysed as controls, and blank values were determined by using distilled water as the test solution.

Free amino acid content of strain NCDO 1250. The strain was grown in the defined medium A as described earlier but with (*a*) asparagine omitted, (*b*) aspartic acid omitted, (*c*) asparagine and aspartic acid omitted but with Tryptone (Oxoid 5 mg./ml.) added. Extracts of washed organisms were made in 10 % (v/v) acetic acid and chromatograms prepared from the extracts by the method of Mattick, Cheeseman, Berridge & Bottazzi (1956).

RESULTS

Growth of strain NCDO 1250 *in medium A and medium B.* Good growth was obtained in defined medium A which contained a mixture of 19 amino acids and an ammonium salt. In contrast, growth did not occur under aerobic or anaerobic conditions in the partially defined medium B in which the amino acid mixture was replaced by acid-hydrolysed casein + tryptophan, cysteine, tyrosine, phenylalanine and histidine (Table 1), even in the presence of 1 μg. folinic acid/ml. medium. However, growth did occur under both conditions of incubation when 0·5 % (w/v) Tryptone (Oxoid; a tryptic digest of casein) was added. These observations suggested that medium B was deficient in some nitrogenous component and accordingly the amino acid requirements of the strain were studied.

Table 1. *Composition of double strength media*

Medium A

Glucose	50 g.	DL-Alanine	400 mg.
Sodium acetate	40 g.	L-Arginine	400 mg.
NH_4Cl	6 g.	L-Aspartic acid	200 mg.
Salts A (Snell & Strong, 1939)	12 ml.	L-Asparagine	800 mg.
Salts B (Snell & Strong, 1939)	10 ml.	L-Cystine	100 mg.
Tween 80	2 ml.	L-Glutamic acid	600 mg.
Adenine, guanine, uracil, xanthine, each	20 mg.	Glycine	200 mg.
Thiamine hydrochloride	1000 μg.	L-Histidine	100 mg.
Pyridoxal ethyl acetal hydrochloride	2000 μg.	DL-Isoleucine	500 mg.
		DL-Leucine	500 mg.
Ca-D-pantothenate	1000 μg.	L-Lysine	400 mg.
Riboflavin	1000 μg.	DL-Methionine	200 mg.
Nicotinic acid	2000 μg.	DL-Phenylalanine	200 mg.
p-Aminobenzoic acid	200 μg.	L-Proline	200 mg.
Biotin	2 μg.	DL-Serine	100 mg.
Folic acid	20 μg.	DL-Threonine	400 mg.
		DL-Tryptophan	80 mg.
		L-Tyrosine	200 mg.
		DL-Valine	500 mg.

Volume to 1000 ml. pH 6·8.

Medium B

Medium B had the same composition as medium A except that 10 g. vitamin-free acid hydrolysed casein (Difco or Allen and Hanbury's), 80 mg. tryptophan, 200 mg. cystine and 400 mg. each tyrosine, phenylalanine and histidine were used in place of the amino acid mixture.

Amino acid requirement of strain NCDO 1250. Omission of the following amino acids (singly) from medium A prevented growth of strain NCDO 1250: arginine, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine. In contrast, no effect on growth was observed when alanine, aspartic acid or lysine was omitted. The omission of asparagine, however, decreased growth by some 50 %.

The amino acid deficiency of medium B. Chromatograms prepared from the Allen and Hanbury's acid-hydrolysed casein indicated that, as compared with medium A, medium B was deficient only in methionine and asparagine. The addition of either or both of these amino acids showed that asparagine was the limiting growth factor. In contrast, chromatograms of Tryptone showed the presence of asparagine. By chemical analysis 1 mg. Tryptone was found to contain 0·08 mg. asparagine and a negligible amount of glutamine. In medium B the growth responses to Tryptone and asparagine were similar (Fig. 1) and indicated that 1 mg. Tryptone was equivalent in activity to about 0·15 mg. asparagine.

Specificity of the requirement of strain NCDO 1250 for asparagine. The growth activity of asparagine in medium B was not replaced by aspartic acid at 25–200 μg./ml. or by glutamine (1–500 μg/ml.) or glutathione (1–500 μg./ml.). In the absence of both asparagine and aspartic acid from the defined medium A no growth occurred until either one or other of the acids was replaced. The growth response to asparagine (aspartic acid absent) was greater than that to aspartic acid (asparagine absent) at the same concentration by weight (0·1 or 0·4 mg./ml.). Increasing the glutamic acid up to 0·9 mg./ml. had no effect on growth.

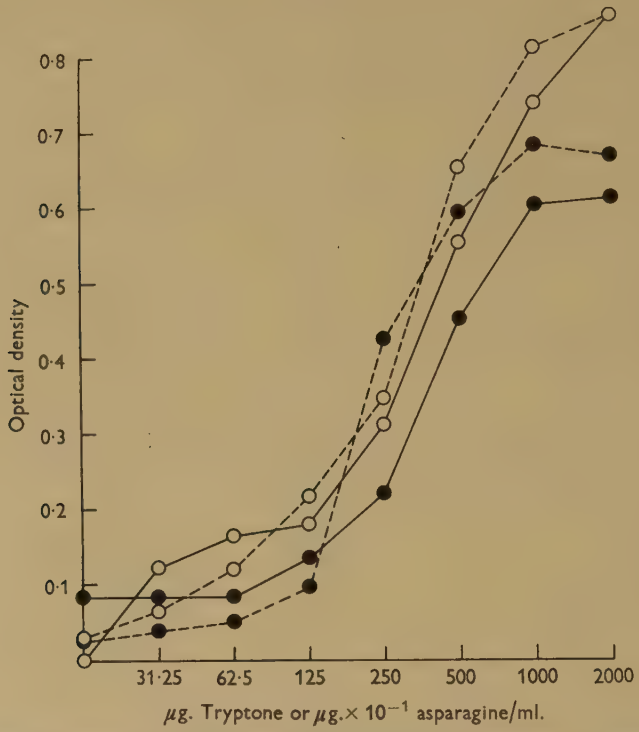

Fig. 1. Growth response of Pediococcus NCDO 1250 to Tryptone or asparagine after 48 hr. in medium B at 30°. ●—● = +asparagine aerobically with ascorbic acid; ●---● = +asparagine anaerobically; ○—○ = +Tryptone aerobically with ascorbic acid; ○---○ = + Tryptone anaerobically.

Effect of asparagine on the amino acid pool. The amino acids found in the extracts of organisms grown in the defined medium A when either asparagine or aspartic acid was omitted, or with both omitted and tryptone added, were qualitatively and quantitatively similar. There were two outstanding features. First, no asparagine could be detected in the organisms even when it had been added to the medium, and, secondly, even when aspartic acid was omitted from the medium, it could still be found in the organisms in undiminished concentration.

DISCUSSION

The use of asparagine in bacteriological media is widespread. In media based on acid-hydrolysed casein it has been reported to stimulate the growth of lactobacilli (Evans & Niven, 1951) and, together with glutamine, of *Streptococcus lactis* (Niven, 1944). Asparagine also promoted the growth of butyric acid bacteria in corn mash but aspartic and glutamic acids were equally effective (Tatum, Peterson & Fred, 1935). Our finding that the Pediococcus strain NCDO 1250 in acid-hydrolysed casein medium had a requirement for asparagine which could not be replaced by glutamine or aspartic acid is similar to that of Niven (1944) for certain of his strains of *Streptococcus lactis*. However, when the casein hydrolysate was replaced by a mixture of amino acids the requirement of the Pediococcus for asparagine was satisfied by aspartic acid + an ammonium salt. It seemed possible that the requirement for asparagine in acid-hydrolysed casein medium was due to an imbalance of amino acids in the medium. For example, the large amount of glutamic acid in acid-hydrolysed casein might inhibit the transport of aspartic acid into the cell (Gale & van Halteren, 1951). However, no support for this hypothesis was obtained. Growth was not promoted when the concentration of aspartic acid was increased nor did high concentrations of glutamic acid inhibit growth in the defined medium.

Stokes, Koditschek, Rickes & Wood (1949) suggested a relationship between asparagine and strepogenin activity. In agreement with this, a major part of the stimulatory activity of Tryptone for the Pediococcus in the acid-hydrolysed casein medium could be attributed to its asparagine content. Asparagine peptides in the Tryptone would not be estimated by the procedure used and might account for the remaining activity (Miller & Waelsch, 1952). The precise role of asparagine in the metabolism of the Pediococcus strain examined remains to be established. Examination of the amino acid pool showed that asparagine was not concentrated there, suggesting that it was rapidly metabolized to provide as one of its functions the aspartic acid requirement of the cell. Whether under certain conditions it is more effective than aspartic acid because it is more easily transported into the cell remains to be determined.

The authors wish to thank Dr H. L. I. Gunther (Queen Elizabeth College, London University) for supplying the strain used in the investigation. They also thank Dr M. E. Sharpe (National Institute for Research in Dairying) for permission to publish the recipe of the tomato glucose broth.

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Comparative Carbohydrate Metabolism and a Proposal for a Phylogenetic Relationship of the Acetic Acid Bacteria

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SUMMARY

The oxidation of 20 carbohydrates and derivatives by 45 strains of acetic acid bacteria, representing most of the known species, was studied and correlated with the enzymic constitution of the organisms. The strains of the mesoxydans group of Frateur and the acetate-oxidizing Gluconobacters oxidized the greatest variety of substrates and contained the most complex enzyme system. The strains investigated could be arranged in two main lines, each one with a stepwise decreasing gradation of oxidative properties. In contrast to the expectations evoked by their names, the strains of the 'oxydans' group had only limited oxidative powers and the 'peroxydans' bacteria even less so. The enzymic mechanism of the catabolism of several carbohydrates was discussed. It is proposed to split the acetic acid bacteria into two biotypes: *Gluconobacter oxydans* and *Acetobacter aceti*, and to consider the existing species as varieties within the two main types. An approach to the phylogenetic interpretation of the intermediary carbohydrate catabolism of these bacteria is discussed.

INTRODUCTION

The present classification of bacteria is nearly always a system for grouping together apparently closely related strains and a useful key for the identification of unknown bacteria. The objections against the basis of this system, of which *Bergey's Manual of Determinative Bacteriology* (1957) is the standard compilation, have been voiced many times (van Niel, 1946, 1953; Skerman, 1949; Winogradsky, 1952; Sneath, 1957*a*) and need not be repeated here. The status of bacterial classification is still far removed from the situation in the taxonomy of higher plants and animals, for it is no reflexion of bacterial phylogeny. The considerations of van Niel (1946, 1953) on this problem and his views on a re-organization of our knowledge of the bacterial world in a natural relationship (adansonian relationship (Adanson, 1763) where morphological, physiological, biochemical, enzymic, ecological, immunological and other properties all have equal value) may open the way to an understanding of its phylogeny. To obtain this goal, a file-index with the complete properties of existing strains would be required, which is a task beyond practical possibilities for most investigators. Nevertheless, a first step in this direction has been made by the analysis with an electrical computing machine of many properties of Chromobacterium (Sneath, 1957*b*; Lysenko & Sneath, 1959) and of many bacteria from the (British) National Collection of Type Cultures (Sneath & Cowan, 1958).

A few years ago we began a study of the comparative carbohydrate biochemistry as a possible basis to show a natural relationship and phylogeny of the species within the group of 'acetic acid bacteria'. These bacteria are well suited for this kind of investigation because their species differentiation is mainly based on their carbohydrate metabolism. In Frateur's system of classification (Frateur, 1950) all the strains are arranged in four groups with the characters summarized in Table 1.

Table 1. *Summary of the properties of the four groups of acetic acid bacteria according to Frateur* (1950)

Group	Catalase	CO_2 production from acetic and lactic acids	Ketogenesis (glycerol to dihydroxyacetone; mannitol to fructose; glucose to 5-keto-gluconate)	Oxidation of glucose to gluconic acid
Peroxydans	−	+	− or very weak	−
Oxydans	+	+	− or very weak	+ (− for *A. ascendens*)
Mesoxydans	+	+	+	+
Suboxydans	+	−	++	++

Extensive investigations in our laboratory of the enzymic equipment of many strains have revealed that often only a few enzymes are responsible for the oxidative properties of intact organisms and that the interpretation of the Warburg experiments was often surprisingly simple and clear-cut in terms of the enzymic outfit of the organisms. Thus it was invariably found that strains which consumed 0·5 mole O_2/mole glucose, oxidized this substrate quantitatively to gluconate with an oxidosome-linked glucose oxidase or a soluble coenzyme-linked glucose dehydrogenase, or both. (Oxidosomes are oxidase-bearing, ultra-microscopic fragments of the 'ghost' or cytoplasmic membrane (De Ley, 1960).) The same applied to many other substrates (see below). Also, several substrates were oxidized by two pathways, one of them being the hexose monophosphate oxidative cycle (also called Horecker cycle, pentose phosphate cycle or shunt), the other usually a non-phosphorylative mechanism. In many cases the relative importance of these two mechanisms could easily be calculated (see, for example, De Ley & Schell, 1959*a*). The study of the oxidative capacities of acetic acid bacteria had thus a considerable advantage over most other bacteria, e.g. the Enterobacteriaceae and others, where the results of Warburg experiments do not allow conclusions about the pathways involved. Resting acetic acid bacteria have been used several times for qualitative studies (Bertho, 1932; Cozic, 1933; Kondo & Ameyama, 1958) or quantitative studies on oxygen uptake. We reported on *Acetobacter peroxydans* (De Ley, 1958*a*), the type species of the 'peroxydans' group, on *A. aceti* (De Ley & Schell, 1959*a*) from the 'mesoxydans' group, on *Gluconobacter suboxydans* (De Ley & Stouthamer, 1959) and on *G. liquefaciens* (De Ley & Dochy, 1960*a*, *b*). Several other species were studied by Stouthamer (1959, 1960). Yet we felt that the potentialities of this method had not been exploited to their full extent. In the present paper we wish to clarify in part this situation by summarizing some of our results with the 45 strains of our collection, which represents most of the known species of acetic acid bacteria. We preferred to use authentic strains which have been described and named by other authors, instead of isolating fresh ones, since the former have often been the origin for the definition of new species, later used in the construction of several systems of classification (Henneberg, 1909; Lafar, 1914; Hermann & Neuschul, 1931; *Bergey's Manual*, 1957; Frateur, 1950). The recent papers by Shimwell (1959) and Shimwell & Carr (1959) rejecting the validity of most of the species of the acetic acid bacteria also induced us to submit this report. The fundamental differences between the interpretation of Shimwell and our own on the relationship amongst these bacteria are discussed below.

METHODS

Organisms. The origin of the strains used are mentioned in Table 2. Upon arrival all the strains were checked for purity by plating on a medium containing (w/v) 10 % glucose, 3 % $CaCO_3$, 1 % yeast extract (Difco) and 2·5 % agar, and were incubated for several days at 30°. Most of the cultures were pure. In some cases mixtures of colony types were seen. From those the strain, corresponding to the name on the label, was isolated. Pure strains were stored on agar slopes of the same medium at 4° and transferred bi-monthly. The purity of the stock cultures was checked several times a year by plating on several media. Occasionally, the stock cultures were also preserved on beer solidified with 12 % (w/v) gelatine. These cultures have been used in our laboratory for about 3 years and in most cases no obvious change in their properties was noticed, which is at variance with the results of Shimwell (1957, 1959) and Shimwell & Carr (1959).

Mass cultures. The bacteria from a 2- or 3-day-old culture on a slope were suspended in sterile 0·01 $\text{M-KH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$ buffer (pH 6·0) and used to inoculate Roux flasks of the same glucose medium as above. In some experiments (see below) other sugars in 5 % (w/v) concentration were used instead of glucose. The bacteria were grown for 2–3 days at 30°, harvested from the agar surface with 0·01 M of the same buffer (pH 6·0) with the aid of a magnetic stirrer, washed at least twice and finally suspended at a concentration of 100 mg. (wet wt.) living bacteria/ml. of the same buffer. These suspensions were used at once for the Warburg and other experiments. *Acetobacter ascendens*, strain A, was unable to grow on glucose; it was therefore grown on beer solidified with 12 % (w/v) gelatine and incubated at 20° for 2–3 days.

Warburg experiments. These were carried out in the conventional apparatus at 30°. The main vessel contained 50–100 mg. wet wt. bacteria, suspended in 1·8 ml. 0·02 M-phosphate buffer (pH 6·0). The side arm contained 10 μmole of the substrate in 0·1 ml. and the central well 0·1 ml. of 20 % (w/v) KOH. The oxygen uptake was followed until the reaction was over. When the oxidation was slow, it was followed up to 5 hr. The results were corrected for endogenous respiration, which was usually nearly negligible. With some strains the endogenous oxygen uptake remained nearly zero for 2–3 hr. and then increased logarithmically to 20 or more μmole O_2 after 5 hr. It appeared to be a spontaneous autolysis of some of the cells, providing oxidizable substrates for the living ones.

Fermentation experiments. A Kluyver type fermentometer (Kluyver, 1914) was filled with pure mercury. One ml. of a 0·033 M-phosphate buffer (pH 6·0) containing 100–500 mg. wet wt. living bacteria and 0·5 mmole glucose was introduced into the upper part of the closed arm and the apparatus incubated at 30° for at least

Table 2. *Origin and specification of the strains, mentioned in this paper*

Collection (name under which the organism was received)	Strain no. used in this paper	Generic name used in this paper
The National Collection of Industrial Bacteria (NCIB), Torry Research Station, Aberdeen		
Acetobacter aceti (Kützing) Beijerinck	8554	*Acetobacter*
A. acetosus (Henneberg) Bergey *et al.*	2224	*Acetobacter*
A. ascendens (Henneberg) Bergey *et al.*	4937	*Acetobacter*
A. ascendens (Henneberg) Bergey *et al.*	8163	*Acetobacter*
A. capsulatus Shimwell	4943	*Gluconobacter*
A. kuetzingianus (Hansen) Bergey *et al.*	3924	*Acetobacter*
A. melanogenus Beijerinck	8086	*Gluconobacter*
A. mesoxydans Frateur	8622	*Acetobacter*
A. mobilis Tosic & Walker	6428	*Acetobacter*
A. pasteurianus (Hansen) Beijerinck	8856	*Acetobacter*
A. peroxydans Visser 't Hooft	8618	*Acetobacter*
A. rancens Beijerinck	8619	*Acetobacter*
A. suboxydans Kluyver & de Leeuw	3734	*Gluconobacter*
A. turbidans Cosbie, Tosic & Walker	6424	*Acetobacter*
A. viscosus Shimwell	8131	*Gluconobacter*
A. xylinum (Brown) Holland	8747	*Acetobacter*
Bacterium vini acetati Henneberg	4939	*Acetobacter*
Bacterium xylinoides Henneberg	4940	*Acetobacter*
Laboratory for Microbiology, Technological University, Delft, Netherlands		
Acetobacter aceti var. *muciparus*	5	*Acetobacter*
A. ascendens (Henneberg) Bergey *et al.*	A	*Acetobacter*
A. lovaniensis Frateur	13	*Acetobacter*
A. melanogenus Beijerinck	16	*Gluconobacter*
A. mesoxydans var. *saccharovorans*	4	*Acetobacter*
A. paradoxus Frateur	30	*Acetobacter*
A. pasteurianus (Hansen) Bergey *et al.*	11	*Acetobacter*
A. rancens Beijerinck	15	*Acetobacter*
A. suboxydans Kluyver & de Leeuw	10	*Gluconobacter*
A. xylinum (Brown) Holland	12	*Acetobacter*
A. xylinum (Brown) Holland	25	*Acetobacter*
Dr I. Mehlman, Department of Food Technology, University of California, Davis, California, U.S.A.		
Acetobacter rancens Beijerinck (received as *A. peroxydans*)	'Davis'	*Acetobacter*
A. ketogenus	24	*Acetobacter*
Gluconobacter cerinus var. *rosiensis*	22	*Acetobacter*
Acetobacter suboxydans Kluyver & de Leeuw	26	*Gluconobacter*
A. rancens Beijerinck	23	*Acetobacter*
Prof. Asai, Institute for Applied Microbiology, Tokyo, Japan		
Gluconobacter liquefaciens Asai	20	*Gluconobacter*
G. cerinus Asai	21	*Gluconobacter*
Prof. J. Frateur, Laboratory for Microbiology, the University, Louvain, Belgium		
Acetobacter aceti (Kützing) Beijerinck	Ch 31	*Acetobacter*
Dr J. G. Carr, Department of Agriculture and Horticulture, the University, Bristol		
Acetobacter estunensis Carr	E	*Acetobacter*
Dr A. J. Stouthamer, Laboratory for Microbiology, the University, Utrecht, Netherlands		
Acetobacter suboxydans Kluyver & de Leeuw	SU	*Gluconobacter*

3 days. The amount of gas produced was read every day. Fermentations were also carried out in the Warburg apparatus in a CO_2 or N_2 atmosphere, with different amounts of glucose or fructose.

The detection of enzymes. As reported in 'Results', this was carried out by the methods described or referred to in previous papers (De Ley, 1954; De Ley & Verhofstede, 1957; De Ley, 1958*a*; De Ley & Schell, 1959*a*, *b*; De Ley & Dochy, 1960*a*, *b*; De Ley & Defloor, 1959).

RESULTS

Forty-five strains were studied. The properties of *Acetobacter peroxydans*, as previously reported (De Ley, 1958*a*) were included for comparison. The species names of the strains, as received from the different laboratories, were used unchanged. In accordance with Leifson (1954) we retained the generic name of *Acetobacter* for the peritrichous or non-flagellated strains of the mesoxydans, oxydans and peroxydans groups of Frateur (1950). For the polarly flagellated bacteria, most of which belong to the suboxydans group of Frateur, we adhered to the name of *Gluconobacter*, originally proposed by Asai (1934, 1935), for reasons to be mentioned below.

Because the bacteria were nearly always grown on glucose, the shape of the oxidation curves in the Warburg experiments might give an indication about the constitutive or inducible nature of the enzymes concerned in the metabolism of the other substrates.

Metabolism and oxidation of glucose

All the strains of our collection could be sharply divided into three groups a, b and c.

Group a. *Acetobacter ascendens*, strain A, *A. paradoxus* 30 and the strains of *A. peroxydans* did not attack glucose at all. We detected neither glucose oxidase nor glucose dehydrogenase. For *A. ascendens*, strain A, this explains the original description of Henneberg (1898), who noticed that this species was unable to make acid when growing on glucose media. Other so-called *A. ascendens* strains, e.g. NCIB 4937 and NCIB 8163, oxidized glucose with the uptake of 0·5 or more mole O_2/mole substrate. If we adhere to the definition of Henneberg and to the above results with strain A, we cannot include these strains NCIB 4937 and NCIB 8163 in the species *ascendens* but have to relegate them to another one.

Group b. Some strains oxidized glucose only with the uptake of 0·5 mole O_2/mole substrate. Here belong *Acetobacter rancens* 15, 23 and 'Davis', *A. pasteurianus* 11 and 8856, *A. kützingianus* 3924, *A. rancens* var. *turbidans* 8619 and *A. ascendens* 8163. The other oxidative capacities of these strains were also almost identical. Frateur (1950) redefined the species *A. rancens*; as an additional criterion to his definition we can now add that this species is able to oxidize glucose only to gluconic acid. On the enzymic level this means that *A. rancens* contains only a glucose oxidase and a glucose dehydrogenase or both, but no hexokinase or other mechanism to introduce glucose into the hexose monophosphate oxidative cycle. *A. lovaniensis* 13 carries out the same reaction with glucose, but is able to grow on ethanol and NH_4^+ as sole carbon and nitrogen source, which differentiates it from *A. rancens*. Both *A. rancens*, as redefined now, and *A. lovaniensis* are unable to use glucose as a carbon source; they grow chiefly at the expense of the yeast extract. The production of gluconate from glucose is an accidental event which has nothing or very little to do with the physiological processes involved in the growth of the organisms. The production of gluconate in both species appears to be a useless reaction, which may possibly be regarded as an atavism, a remnant of a metabolic pathway from other acetic acid bacteria, such as the one we unravelled for *Gluconobacter suboxydans* (De Ley & Stouthamer, 1959). This interpretation would also explain the results of Hermann & Neuschul (1931) who found that many strains of acetic acid bacteria, while growing on glucose, converted this substrate nearly quantitatively into gluconate.

Group c. All the other strains of the collection oxidized glucose with an uptake of more than 0·5 mole O_2/mole substrate: from 1·2 for *Acetobacter estunensis* E to 4·9 O_2 for *A. mesoxydans* var. *saccharovorans* 4. On the whole most strains took up 1–3 mole O_2/mole substrate, which was far below the theoretically expected value of 6. The explanation for this phenomenon was given in a previous paper (De Ley & Schell, 1959*a*). Indeed, glucose was metabolized by two independent pathways, which competed for the substrate; one was the oxidation to gluconate and the other the hexokinase, followed by the hexose monophosphate oxidative cycle. If the former mechanism alone was active, an uptake of 0·5 mole O_2/mole glucose was expected; with the latter alone, 6 mole O_2/mole glucose. Intermediate O_2 uptake between these two extremes allows one to calculate how much of the glucose was oxidized by each pathway. We found (De Ley & Schell, 1959*a*) that *A. aceti* Ch 31 converted about 80 % of the glucose into gluconate, the remaining quantity being used as carbon and energy source. This calculation was corroborated by chemical analysis of the end products. By applying similar calculations to the strains of the present paper, which did not oxidize gluconate further, it was found that, for example, *A. vini acetati* 4939 converted 84 % of the glucose to gluconate, *A. estunensis* E about 87 %, etc. The remaining glucose was thus used by way of the hexose monophosphate oxidative cycle, which explains the irisation which occurs when these strains grow on the glucose–$CaCO_3$–yeast extract–agar medium. For most of the strains of the mesoxydans group and for Gluconobacter, these calculations could not be used, because ketohexonates were also formed.

Efficiency of glucose as a carbon and energy source for growing cells. For most of the strains the yield (wet weight) of organisms/Roux flask was determined. Figure 1 shows that there was a clear correlation between the yield of organisms and their ability in a resting state to oxidize glucose. The higher the O_2 uptake/mole substrate (that is the more glucose was consumed via the hexose monophosphate oxidative cycle), the higher the yield of the organisms. Organisms which oxidized glucose only to gluconate grew poorly as compared to the others, and all belonged to the species *rancens* as redefined above, which also strengthened the former conclusions. Again, this way of interpretating the results applied best to the bacteria of the oxydans group. For the strains of the mesoxydans group and for Gluconobacter, the excess O_2 uptake over 0·5 mole O_2 did not represent a true measure of the disappearance of glucose by way of the hexose monophosphate oxidative cycle. Yet, as a whole, the same general tendency was observed.

Glycolysis and the fermentation of glucose in the acetic acid bacteria. Neuberg & Simon (1928*a*, *b*) and Simon (1930) found that *Acetobacter ascendens*, *A. pasteurianus* and *Gluconobacter suboxydans* fermented glucose with the formation of about equal amounts of ethanol and CO_2. From a recalculation of their results it may be seen that only part of the glucose fermented was accounted for by these end products. The remainder (usually 50–80 %) was apparently converted into unidentified end products. Simon (1930) withdrew the previous results and reported that indeed all of the glucose was accounted for as ethanol + CO_2. Why then was glucose not fermented to completion? The answer may be deduced from an experiment of Simon, in which he showed that ethanol appeared to inhibit the fermentation. These authors also described the presence of enzymes of glycolysis in these bacteria. Their conclusion, that acetic acid bacteria contained an active glycolytic mechanism, found its way in the literature (see, for example, Thimann, 1955). In fact, the results of Neuberg & Simon are difficult to understand. Indeed, the typical *A. ascendens* cannot oxidize glucose at all; it contains neither glucose oxidase nor hexokinase. *A. pasteurianus* contains no hexokinase and oxidizes glucose only to gluconate. How then could these strains ferment glucose? A tentative explanation is that the strains used were neither ascendens nor pasteurianus. In the hope of detecting either the unknown end products or the mechanism of glucose fermentation by using labelled substrates, we repeated the experiments of Neuberg & Simon, but were unable to confirm their results. Most of the strains of our collection were used with the Kluyver fermentometer. In most cases only a trace of gas was formed. The gas production rarely exceeded 10 % of the theoretically expected amount of CO_2

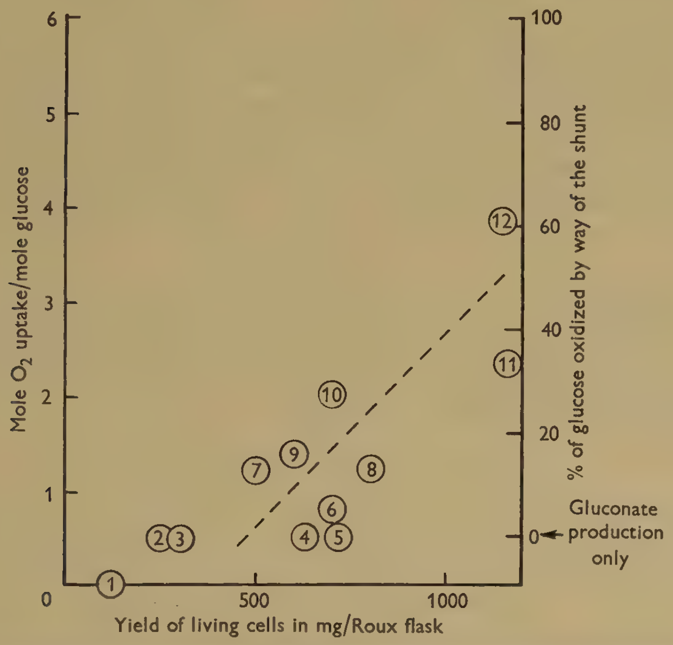

Fig. 1. The relationship between the yield of living cells (wet weight) from the oxydans group after 3 days of growth on the glucose medium in Roux flasks and the final O_2 uptake with glucose as a substrate in the Warburg with the same cells in a resting state. Strains used: 1, *Acetobacter ascendens* A; 2, *A. lovaniensis* 13; 3, *A. kützingianus* 3924; 4, *A. pasteurianus* 8856; 5, *A. pasteurianus* 11; 6, *A.* '*ascendens*' 8163; 7, *A. estunensis* E; 8, *A.* '*ascendens*' 4937; 9, *A. vini acetati* 4939; 10, *A. mobilis* 6428; 11, *A. turbidans* 6424; 12, *A. acetosus* 2224.

(22·4 ml.) according to an alcoholic fermentation. At the end of the reaction 1 ml. of 20% (w/v) KOH was introduced and it appeared that only about three-quarters of the gas was CO_2. The nature of the residue was not assayed; only about 0·5 ml. or less was available. It could not have been due to an air leak, since the gas was always under a slight excess pressure, due to the level of the mercury in the open tube. In some cases more than 10% of the expected amount of CO_2 was formed, but it could invariably be shown that the suspension had become contaminated during storage in the fermentometer, generally with coliform organisms. Neither could gas formation be observed in the anaerobic Warburg experiments, even when they were continued for 5–7 hr. and with varying concentrations of sugar. Prof. J. Frateur kindly informed us (in 1959) that he had been unable to corroborate the results of Neuberg & Simon. Stouthamer (1960) also found no fermentation with *Gluconobacter liquefaciens*. Kitos *et al.* (1958) reported that in *G. suboxydans* the shunt accounted for the bulk of the oxidation. The general conclusion from all these results appears to be that only a few acetic acid bacteria may contain a very weak glycolytic system, that the claims of Neuberg & Simon have on the whole not been confirmed, and that it seems desirable to erase this opinion from the literature.

Oxidation of gluconate

Most of the strains of the oxydans group of Acetobacter were unable to oxidize gluconate. Nearly all the strains of the mesoxydans group and of *Gluconobacter* attacked this substrate vigorously. Only *Acetobacter aceti* 8554 and Ch 31, *A. aceti* var. *muciparus* 5 and *A. ketogenus* 24 attacked it very weakly.

Oxidation of 2- *and* 5-*ketogluconate*

None of the strains of the oxydans group were able to attack either 2- or 5-ketogluconate. In the mesoxydans groups of Acetobacter and in Gluconobacter, the oxidation occurred at random and could not be used for taxonomic purposes. The oxidative abilities for these substrates denoted merely the presence or absence of enzyme systems, described previously (De Ley, 1958*a*; De Ley & Defloor, 1959; De Ley & Stouthamer, 1959), which appeared to be arbitrarily distributed throughout the representatives of the latter groups. It should be stressed, however, that the Warburg method is unable to indicate the presence of weak enzymes. This explains the apparent discrepancy between our results and those of Frateur, Simonart & Coulon (1954). These authors found by paper chromatography that several strains of the oxydans group were able to form 2-ketogluconate when grown on glucose for up to 30 days. In the Warburg apparatus an uptake of 1 μmole O_2/5 hr./100 mg. wet wt. bacteria would surely be regarded as negligible. Yet it can be calculated that this low enzyme activity would be sufficient to allow the production of 2·9 mmole or 617 mg. calcium 2-ketogluconate from glucose/month/g. wet wt. bacteria.

The oxidation of mannitol and fructose

Most strains of the oxydans group of Acetobacter oxidized neither mannitol nor fructose; some strains oxidized both very slowly. Nearly all the strains of the mesoxydans group of Acetobacter and of Gluconobacter oxidized both substrates. Arcus & Edson (1956) showed a DPN-linked mannitol dehydrogenase in *G. suboxydans* and we found a TPN-specific dehydrogenase in *A. aceti* Ch 31 (De Ley & Schell, 1959*a*). Fructose, which was formed or excreted outside the cell, became unavailable. We explained this by postulating a fructose permease. Mannitol, which was oxidized to fructose within the cells, was further metabolized. We have encountered similar situations in *G. viscosus* 8131 and *G. capsulatus* 4943. From all the strains investigated from the latter groups, only *A, mesoxydans* 8622, was unable to oxidize mannitol. It thus appears that the oxidation of mannitol by resting cells might be used as an additional criterion to decide whether a strain belongs in the oxydans or peroxydans groups of Acetobacter (mannitol negative), or belongs in the mesoxydans group of Acetobacter or in Gluconobacter. Fructose oxidation is less suitable for this purpose.

The oxidation of mannose, galactose and pentoses

Most of the strains investigated were able to oxidize mannose, galactose and pentoses to the corresponding sugar acids. Nearly all the strains except a few representatives of the oxydans group of Acetobacter oxidized mannose with the uptake of 0·5 mole O_2/mole substrate, presumably to mannonic acid. The same is also true for the oxidation of galactose and xylose, from which the corresponding galactonic and xylonic acids were formed. Only *Gluconobacter cerinus* 21 and *G. liquefaciens* 20 oxidized galactose with the uptake of 1·0 or more mole O_2/mole substrate, possibly to the 2-ketogalactonate stage or beyond. *G. cerinus* 21 oxidized xylose with the uptake of 0·95 mole O_2/mole substrate. About half of the strains from the oxydans group of Acetobacter did not attack these substrates. There was a remarkable correlation between the oxidation of both substances: strains which oxidized galactose also converted xylose into xylonic acid and strains which did not attack galactose did not oxidize xylose. This might suggest that the same enzyme was possibly responsible for the oxidation of both substrates.

Ribose and L-arabinose were never oxidized beyond the stage of the corresponding pentonates. We showed previously that all the above substrates are oxidized by oxidases, located on the cytoplasmic membrane (De Ley & Dochy, 1960*a*, *b*). D-Arabinose was not oxidized by any of our strains.

Two main conclusions can be drawn from these results. (i) Mannose, galactose, xylose, L-arabinose and ribose are unsuitable carbon sources for the growth of acetic acid bacteria. On the other hand, these bacteria show excellent promise for the eventual industrial production of these sugar acids. The corresponding enzymes effect an oxidation which has no or very little physiological meaning for the growth of the organisms, which, however, oxidize the substrate when it is present, because they happen to possess the oxidase. The situation is similar to the oxidation of glucose by *Acetobacter rancens* and *A. lovaniensis*. This possibility was supported by the following experiments. *A. turbidans* 6424 and *A. xylinum* 12 were grown on a medium containing either 5% galactose or 5% xylose, 3% $CaCO_3$, 1% yeast extract (Difco) and 2·5% Bacto-agar (Difco). These organisms were harvested and washed as usual. The yields of organisms, when grown on glucose, galactose, or xylose were nearly identical, showing that indeed they did not adapt by growing on the latter two substrates and that they were unable to use them as carbon or energy sources. Furthermore, organisms grown on either of the three substrates oxidized them in the Warburg at the same rate and to the final stage of galactonate and xylonate. (ii) The ubiquitous oxidative capacity for mannose, galactose and the pentoses in the mesoxydans group of Acetobacter and in Gluconobacter, and the distribution in the oxydans group of Acetobacter made this a poor taxonomic character.

The oxidation of maltose and saccharose

The ability to oxidize maltose and sucrose was distributed at random over the strains examined. Again the oxydans group of Acetobacter was the poorest. About half of the strains of the mesoxydans group of Acetobacter and of Gluconobacter was able to oxidize either both or one of these substrates. Therefore it seemed appropriate to abolish the varieties 'maltovorans' and 'saccharovorans', as used in the system of Frateur. Lactose and raffinose were not oxidized by any of our strains.

Oxidation of glycerol

Glycerol was oxidized nearly to completion by all strains tested, except by *Acetobacter peroxydans*, *A. ascendens* 4937 and hardly by *A. vini acetati* 4939. It is probably an excellent carbon and energy source. An O_2 uptake of at least 2 mole O_2/mole glycerol was quite common. In some cases an induction period was noted, possibly pointing to the formation of an inducible enzyme (glycerokinase) in the enzyme chain.

Oxidation of i-erythritol

i-Erythritol seemed to be very promising for use in classification. None of the strains of the oxydans group of Acetobacter were able to attack it, while all the strains of the mesoxydans group of Acetobacter and of Gluconobacter attacked it. A strain which we had received as *G. cerinus* var. *rosiensis* 22 appeared to be the only exception. Closer inspection of its properties showed it to be closely related to either *A. turbidans* 6424 or *A. acetosum* 2224. Very often the oxidation proceeded beyond the erythrulose stage (see Table 3). i-Erythritol was oxidized by an oxidase located on the cytoplasmic membrane (De Ley & Dochy, 1960 *a*, *b*).

The oxidation of D*-lactate, ethanol and acetate*

All the strains of the oxydans, mesoxydans and peroxydans groups of Acetobacter oxidized D-lactate, ethanol and acetate nearly to completion. We described previously the enzymic mechanism of D-lactate and ethanol oxidation in *A. peroxydans* (De Ley & Schell, 1959 *a*). Unpublished experiments carried out with several other species, such as *A. aceti* Ch 31, *A. ascendens* A, showed that this type of mechanism is a general one in all species investigated. The ability to use D-lactate and ethanol as carbon and energy sources seemed to be the really essential physiological activity for Acetobacter. In several cases an induction period was noticed in the oxidation of acetate. In the polarly flagellate Gluconobacter there appeared to be two distinct groups: (i) *G. liquefaciens* 20, which was able to oxidize ethanol, D-lactate and acetate as already pointed out (Asai & Shoda, 1958; Stouthamer, 1959, 1960; De Ley & Dochy, 1960); (ii) bacteria such as *Gluconobacter suboxydans* 26, 10 and SU, *G. viscosus* 8131, *G. capsulatus* 4943 and *G. melanogenus* 8086 and 16, which were unable to oxidize acetate and oxidized D-lactate and ethanol not beyond acetate. The latter group corresponded to the 'suboxydans' bacteria of Frateur. It may be noted that *G. cerinus* 21, although it did not oxidize acetate, nevertheless oxidized D-lactate and ethanol nearly to completion.

Table 3. *The oxidation of some carbohydrate derivatives by some representative strains of acetic acid bacteria*

Content of the Warburg vessels: see text. The results are expressed as mole O_2 uptake per mole substrate at the end of the oxidations. In some cases the reaction was not yet over after 5 hr. and is marked by the sign >

Strain	Glucose	Sodium gluco- nate	Sodium 2-keto- gluco- nate	Sodium 5-keto- gluco- nate	Mannitol	Fructose	Mannose	Galac- tose	Xylose	Glycerol	i-Ery- thritol	Sodium D-lactate	Ethanol	Sodium acetate
Peroxydans group														
A. peroxydans (8618)	0	0	0	0	0	0	0	0	0	0	0	2·7	2·6	1·4
Oxydans group (22 strains investigated)														
A. ascendens (A)	0	0	0	0	0	0	0	0	0	2·3	0	2·5	2·3	1·8
A. rancens (15)	0·5	0	0	0	0	0	0	0·1	0·2	2·2	0	2·5	> 1·6	> 0·65
A. lovaniensis (13)	0·5	0	0	0	0	0	0·46	0·5	0·5	2	0	> 1·96	> 1·4	> 0·5
A. ascendens (4937)	1·0	0	0	0	0	0	0	0	0	0	0	> 1·7	2·4	> 1·2
A. vini acetati (4939)	1·4	0	0	0	0	0	> 0·2	> 0·3	> 0·3	> 0·3	0	> 2·2	2·4	1·8
A. estunensis (E)	1·2	> 0·3	0	0	0	0	> 0·3	0·4	0·5	2·2	0	2·3	2·35	1·85
A. turbidans (6424)	2·3	> 1·6	0	0	0	0	0	0·4	0·3	2·3	0	2·5	2·8	2·0
A. mobilis (6428)	2·0	> 0·8	0	0	> 0·5	0·2	0·2	0·6	0·4	2·5	0	2·5	2·4	> 1·8
Mesoxydans group (14 strains investigated)														
A. aceti (8554)	1·8	0·2	0	0	> 1·4	> 0·3	0·5	0·5	0·5	2·5	> 0·7	2·7	2·2	1·9
A. mesoxydans (8622)	> 3·0	> 2·1	0	0	0·2	0	0·4	0·5	0·4	2·5	0·3	2·1	2·4	> 1·7
A. mesoxydans (4)	4·9	4·4	> 3·1	> 3·4	> 1·3	> 1·1	0·4	0·6	0·5	2·7	> 1·2	2·9	> 1·7	> 0·7
A. xylinum (8747)	> 3·5	> 3·3	0·6	> 0·4	> 3·3	> 1·7	0·4	0·4	0·5	> 2·1	> 0·8	> 2·1	> 2·0	> 1·1
Suboxydans group (*Gluconobacter*) (9 strains investigated)														
G. capsulatus (4943)	1·4	0	0	0	> 1·9	0	0·5	0·5	0·5	0·9	0·5	0·23	0·9	0
G. suboxydans (26)	3·1	2·5	0	0·1	4·3	> 1·7	0·6	0·5	0·5	1·7	> 1·1	1·0	0·8	0
G. melanogenus (8086)	2·2	1·3	0·5	0	4·8	4·4	0·5	0·5	0·5	> 1·6	0·6	0·7	0·8	0
G. cerinus (21)	4·5	4·0	0·3	> 1·5	3·5	1·0	0·3	0·9	0·9	2·9	2·2	3·0	2·6	0
G. liquefaciens (20)	2·2	1·1	0·5	2·6	> 2·4	1·8	0·6	> 1·0	0·7	2·35	0·5	1·9	1·9	1·4

DISCUSSION

Use of the generic name Gluconobacter. There is convincing evidence in the literature that the acetic acid bacteria can be divided into two distinct groups: (i) the peritrichously flagellate or non-flagellate bacteria of similar physiology, named Acetobacter, as redefined by Leifson (1954); (ii) the polarly flagellate or non-flagellate strains of similar physiology for which either the name Gluconobacter Asai or Acetomonas Leifson amended Shimwell are used. Asai (1934, 1935) realized for the first time the sharp distinction within the acetic acid bacteria and gave generic rank to two groups, *Acetobacter* and *Gluconobacter*, based on several physiological properties. These results were considerably extended by Asai & Shoda (1958). Twenty years after Asai's first papers Leifson (1954) rediscovered the duality of these bacteria from his studies of the type of flagellation and proposed a new genus *Acetomonas* for the polarly flagellate strains which oxidized neither lactate nor acetate. This genus was studied and reviewed by Shimwell & Carr (1959). These genera, *Gluconobacter* and *Acetomonas*, are almost identical and a slight redefinition of the former would suffice to make the latter superfluous. Asai (1934, 1935) and Asai & Shoda (1958) included several strains into *Gluconobacter* which did not oxidize ethanol to acetic acid and were possibly pseudomonads. The exact position of the acetate-oxidizing *Gluconobacter* species has not yet been settled. According to Asai & Shoda (1958) both *G. liquefaciens* and *G. melanogenus* are polarly flagellate. According to Shimwell & Carr (1959) *G. melanogenus* had sparse peritrichous flagella, and Stouthamer (1960) published a not very convincing picture of a supposedly peritrichous *G. liquefaciens*. Pending further investigations, we accept Asai's view. On the evidence of our results about the gradual change in the enzymic make-up within the cluster of strains (see below), it would not seem very probable that an abrupt distinction between polarly and peritrichously flagellate strains would exist. One can expect a gradual transition from one type into the other, where it might often be hard to make an impartial decision. For historical reasons we prefer to use the name Gluconobacter with the redefinition as given by Asai & Shoda (1958), but excluding the strains which are unable to oxidize ethanol to acetic acid.

The oxidative properties of the groups according to Frateur's classification. Frateur's system and nomenclature was built up on the physiological properties of intact organisms. Unfortunately it is a general belief that acetic acid bacteria and in particular those of the oxydans group, possess strongly oxidative properties whereby they oxidize their substrates nearly to completion. This was supposed to be the reason why ketogluconates, fructose, sorbose, dihydroxyacetone, etc. did not accumulate as intermediates from their respective starting compounds. We may quote, for example, Bernauer (1940): 'So sehen wir z.B. bei den Essigbakteriën, dasz manche derselben (wie *A. rancens*) nur in geringe Masse primäre Abbauprodukte anhaüfen. Diese Bakteriënart hat ein sehr starkes Oxydationsvermögen und baut in der Regel gleich bis zu CO_2 ab'. Also Frateur (1950, p. 307) says: 'Le groupe oxydans est caractérisé par un pouvoir oxydatif très brutal. Ses représentants sont capables d'oxyder complètement, jusqu'au stade CO_2 et H_2O, une grande quantité de substrats organiques'. Table 3 shows that, quite opposite to expectation, the bacteria from the oxydans group had very poor oxidizing capacities which were nearly comparable to the representatives from the peroxydans group. On the other hand, the mesoxydans group, which, as the name indicates, was supposed to occupy a position between the oxydans and the suboxydans group, exhibited the greatest diversity of oxidative abilities. The bacteria from the suboxydans group occupied a separate position, since most of them lacked the Krebs cycle. Nevertheless, they oxidized nearly the same range of substrates as the mesoxydans group, and they did it to the same extent. These results agree on the whole with the reports of Stouthamer (1959, 1960). Previously it was shown (De Ley, 1958) that strains of the peroxydans group can oxidize only a few substrates. It can thus be

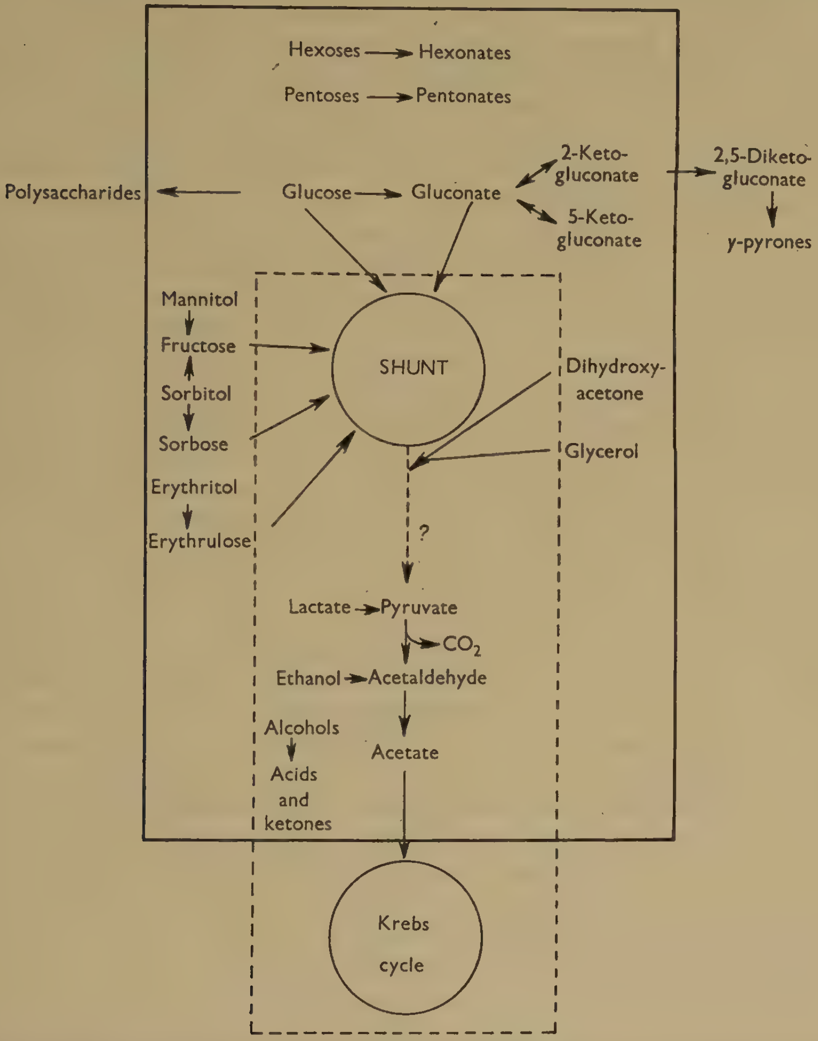
Hexoses → Hexonates
Pentoses → Pentonates
Polysaccharides
Glucose → Gluconate
2-Keto-gluconate
5-Keto-gluconate
2,5-Diketo-gluconate
γ-pyrones
Mannitol
Fructose
Sorbitol
Sorbose
Erythritol
Erythrulose
SHUNT
Dihydroxy-acetone
Glycerol
?
Lactate → Pyruvate
CO2
Ethanol → Acetaldehyde
Alcohols
Acids and ketones
Acetate
Krebs cycle

Fig. 2. Simplified metabolic map of the acetic acid bacteria. The overall picture represents the main part of the metabolism of *Gluconobacter liquefaciens*. Although the latter strain is not known to make polysaccharides, this property was included because it is common to many strains. Two typical extreme divergencies are *G. suboxydans* (metabolic map within the full lines) and *Acetobacter peroxydans* (broken lines).

seen that the nomenclature of the four groups according to Frateur (1950) becomes misleading in view of the better knowledge of the enzymic constitution of the different organisms.

Comparative carbohydrate biochemistry of the acetic acid bacteria. The oxidative behaviour of resting bacteria represents only the overall activity of the enzyme systems which have been studied in previous papers. The core of their intermediary carbon catabolism appears to consist of three essential parts: (i) Hauge, King & Cheldelin (1955), Kitos *et al.* (1958) and Gromet, Schramm & Hestrin (1957) showed the presence of the hexose monophosphate oxidative cycle in *Gluconobacter suboxydans* and *Acetobacter xylinum.* Unpublished work in our laboratory with many other strains has confirmed these results. Stouthamer (1960) detected the same mechanism in *G. liquefaciens.* (ii) The presence of the Krebs cycle enzymes was detected in *A. pasteurianus* (King, Kawasàke & Cheldelin, 1956), in *A. aceti* (Rao, 1955) and in *G. liquefaciens* (Stouthamer, 1960). It seems likely that this mechanism is present in all the acetic acid bacteria, except for the strains of *Gluconobacter* which are unable to oxidize acetate (see, however, *G. cerinus*, above). Rao (1955) found indeed that *G. suboxydans* showed only a few steps of this cycle. (iii) Lactate and ethanol are oxidized to acetate by two pathways; one is effected by a set of oxidosome-linked enzymes (De Ley, 1960) and the other by soluble enzymes. This enzymic mechanism was extensively studied in *A. peroxydans* (De Ley & Schell, 1959*a*) and in many other strains (Schell, 1960). Species differentiation is mainly based on the carbohydrate metabolism of the strains (e.g. polysaccharide formation, ketogenic properties, glucose oxidation, etc.). The enzymic explanation of these differences shows that it is very often only a matter of one or at most a few enzymes, located in the outer regions of the metabolic map (Fig. 2).

The natural relationship of the acetic acid bacteria and a proposal for their phylogeny. As pointed out in the introduction, the ideal approach to the rational classification of bacteria consists in giving equal weight to all the properties exhibited by the strains. Many of these properties are an outward reflexion of the inner enzymic anatomy of the organism and in several cases they represent the truth as little as the shadow of an object tells about the object itself. Within one group of closely related bacteria one can considerably limit the number of properties to be studied, analyse their diverse physiological and biochemical behaviour in terms of their enzymic constitution and compare afterwards their metabolic maps. The comparative biochemical approach might apparently give better results in constructing a natural relationship of bacteria and lead to a model of their phylogeny. The former objective is within reach, because it is a description of an existing situation; the latter objective (phylogeny) can be little more than an hypothesis, because it suggests changes in the course of time, deduced from the present-day state. It may be impossible to construct a complete phylogenetic tree for bacteria from the beginning, because of the great time period involved in their evolution, their short generation time, the enormous possibilities for mutation and because of the lack of evidence of comparative morphology and anatomy, embryology and palaeontology, which have been imperative for our knowledge of the evolution of higher organisms. We can at best hope that the knowledge of the comparative biochemistry of existing bacteria might help to elucidate the latest stages in their evolution. Modern trends in biology allow speculation through the concept of 'regressive evolution'. It is now a generally accepted belief that the most recent stages in the evolution of many higher organisms consist mostly in gradual loss of properties. On the biochemical level there are several examples of consecutive losses of enzymes, accompanying further stages in evolution (Florkin, 1944). The same trend exists in microbiology. For example, Braun (1953) mentioned several possibilities of a regressive physiological evolution in micro-organisms, the most clear-cut being the commonly occurring mutation from prototrophy to auxotrophy and the transition coliform organisms→typhoid bacilli→dysentery bacilli, involving successive losses of fermentative and biosynthetic steps. A bacterial classification constructed in accordance with this concept, in which the strains of a genus are arranged in a sequence of decreasing capacities, might come closest to representing a phylogenetic relationship. This principle is used in Table 3. From there it is a small step to the hypothesis of phylogenetic lines represented in Figs. 3–5. The genera *Acetobacter* and *Gluconobacter* have many features in common, such as the enzymic mechanism of the oxidation of D-lactate and ethanol to acetate, the enzymes of the hexose monophosphate oxidative cycle, but in particular the oxidosome-linked nature of hexose and pentose oxidation, the formation of 2-ketogluconate, the ketogenic characteristics (glycerol to dihydroxyacetone, mannitol to fructose, sorbitol to sorbose, gluconate to 5-ketogluconate, etc.), their acid resistance, etc., that little doubt can remain about their close phylogenetic relationship. In fact many of the quoted properties are so typical for the acetic acid bacteria that they are not encountered in other bacteria, except sometimes in Pseudomonas. One can imagine that both genera evolved from the same pool of ancestors as shown in Fig. 3. In

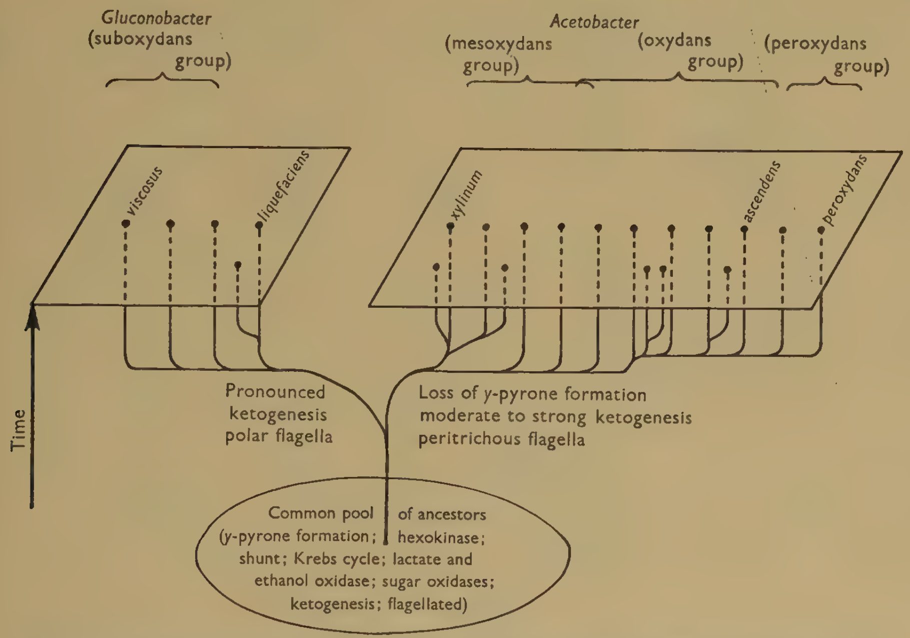
Gluconobacter
(suboxydans group)
Acetobacter
(mesoxydans group)
(oxydans group)
(peroxydans group)
viscosus
liquefaciens
xylinum
ascendens
peroxydans
Time
Pronounced ketogenesis polar flagella
Loss of γ-pyrone formation moderate to strong ketogenesis peritrichous flagella
Common pool of ancestors (γ-pyrone formation; hexokinase; shunt; Krebs cycle; lactate and ethanol oxidase; sugar oxidases; ketogenesis; flagellated)

Fig. 3. Possible origin of the acetic acid bacteria from our collection, based mainly on comparative carbohydrate metabolism. For details see Fig. 4 and 5.

spite of the present uncertainty on the exact position of the acetate-oxidizing *Gluconobacter* species it is clear, according to the above concept, that these strains would be the closest representatives of the ancestral pool of the acetic acid bacteria. From those, or closely related strains, might have evolved in the course of time the presently existing acetic acid bacteria. The relationship between the strains which we studied is represented in Figs. 4 and 5. Both figures represent only some of the 45 strains actually investigated. If a much larger collection had been used, or all the known strains had been included, a much more ramified picture might have emerged.

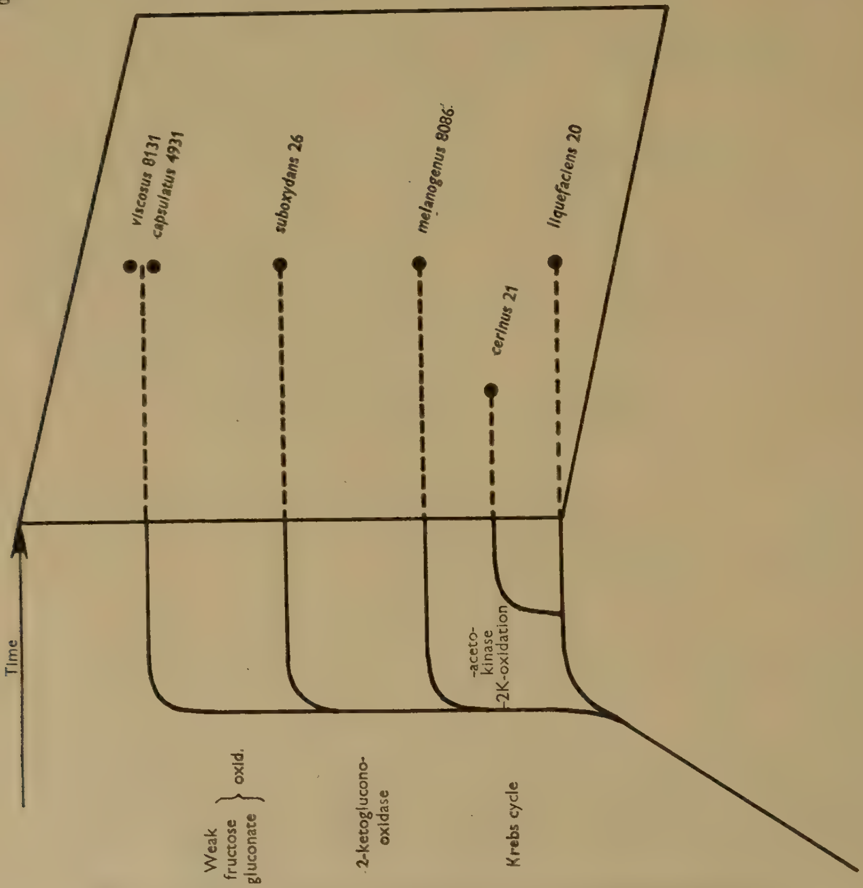
viscosus 8131
capsulatus 4931
suboxydans 26
melanogenus 8086
liquefaciens 20
cerinus 21
-aceto-
kinase
+2K-oxidation
Time
Weak
fructose
gluconate
oxid.
2-ketoglucono-
oxidase
Krebs cycle

Fig. 4. Details of the scheme in Fig. 3. The *Gluconobacter* biotype.

The value of the 'species' concept in the acetic acid bacteria. A considerable number of 'species' of acetic acid bacteria have been described. Frateur simplified the situation by relegating closely related varieties to ten main species. Shimwell (1959) and Shimwell & Carr (1959) followed this attitude to an extreme and established only one species, *Acetomonas oxydans*, for the suboxydans group of Frateur and no type species at all in the genus *Acetobacter*. The use of species names implies that each 'species' is characterized by a number of typical and specific morphological and physiological differences, which set them apart from all the others. Our experience with these bacteria (see Figs. 4 and 5) shows that there are no such abrupt changes in the sequence of strains and that, in each phylogenetic line, they can be arranged in a smooth gradation from the most complex one to the one with the poorest enzymic equipment (from *Gluconobacter liquefaciens* to *G. viscosus* and from *Acetobacter xylinum* to *A. peroxydans*). The differences between a strain and its two immediate neighbours consists at most in one or a few enzymes. With this in mind, it is hard to see where to draw the line in this gradation of properties for defining the limits of a 'species'. The most logical attitude would be to regard the entire cluster of strains (a 'biotype' according to Winogradsky, 1952) from each phylogenetic line as variations of one species and to drop their species names altogether. For sake of clarity and also for historical reasons we shall call the two biotypes *Acetobacter aceti* (Beijerinck, 1898) Beijerinck, 1900, and *Gluconobacter oxydans* (Henneberg) comb.nov. There is apparently one practical disadvantage to this new proposal. This is that within each biotype a considerable variety is encountered: compare, for example, *A. xylinum* and *A. peroxydans*, each being at a different end of the line and yet making part of the same biotype. Yet each strain

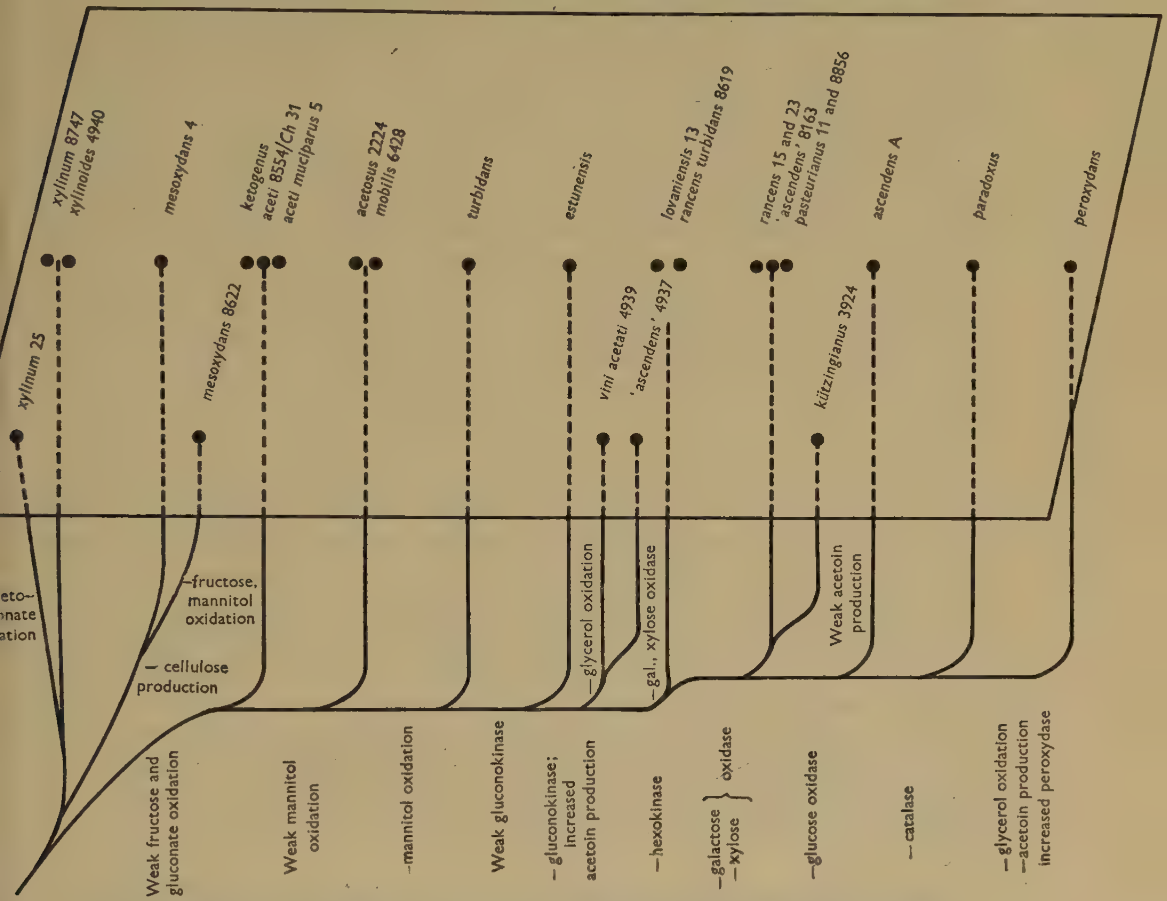
xylinum 8747
xylinoides 4940
mesoxydans 4
ketogenus
aceti 8554/Ch 31
aceti muciparus 5
acetosus 2224
mobilis 6428
turbidans
estunensis
lovaniensis 13
rancens turbidans 8619
rancens 15 and 23
'ascendens' 8163
pasteurianus 11 and 8856
ascendens A
paradoxus
peroxydans
xylinum 25
mesoxydans 8622
vini acetati 4939
'ascendens' 4937
kützingianus 3924
eto-
nate
ation
-fructose, mannitol oxidation
— cellulose production
—glycerol oxidation
—gal., xylose oxidase
Weak acetoin production
Weak fructose and gluconate oxidation
Weak mannitol oxidation
-mannitol oxidation
Weak gluconokinase
—gluconokinase; increased acetoin production
—hexokinase
—galactose
—xylose
oxidase
—glucose oxidase
— catalase
—glycerol oxidation
—acetoin production
increased peroxydase

Fig. 5. Detail of the scheme in Fig. 3. The *Acetobacter* biotype. The property to make polysaccharides was not taken into account, since it is a characteristic easily lost and gained by adaptation and mutation.

requires a label for recognition, whether it be a name or a number. For the sake of clarity and for historical reasons the strains could be labelled as, for example, *A. aceti* (peroxydans), *A. aceti* (xylinum), *Gluconobacter oxydans* (melanogenum), etc., much as our strains now bear a collection number. The previous species names would then be degraded merely to catalogue numbers and indicate a set of enzymic properties. The characteristics for new and unknown strains could be determined according to the criteria of Frateur, flagella staining (possibly electron microscopy) and the Warburg apparatus (O_2 uptake) criteria as in the present paper. The creation of new species names should be discouraged in the future, at least when the new strains are closely related to those already existing.

It might appear that our proposal to abolish species names for the acetic acid bacteria and to substitute two names of biotypes, is merely a confirmation of the conclusions of Shimwell (1959) and Shimwell & Carr (1959). In fact, the proposals are fundamentally different and the conclusions were reached on entirely different grounds. According to Shimwell, the acetic acid bacteria are 'overwhelmingly' unstable, each species mutating spontaneously into another in the laboratory. With such changing properties classification would be impossible; stable strains would not exist. This is contrary to our experience with these bacteria; most of our strains showed unchanged sets of properties over a period of three years. We can thus not accept Shimwell's view of the abnormally high mutability of acetic acid bacteria. Frateur (1950 and personal communication) also emphasized the stability of his strains. Undoubtedly, several authors, including ourselves, have observed spontaneous mutations in this group (but probably not more frequently than with other bacteria). A direct enzymic analysis of the spontaneous change of *Gluconobacter melanogenus* into *G. suboxydans* (De Ley & Stouthamer, 1959) showed that at most two enzymes only were affected. The enzymic interpretation of the 'polysaccharide-less' mutants (*Acetobacter xylinum* to *A. mesoxydans* and *A. pasteurianus* to *A. rancens*) as well as of the other results of Shimwell leads to the same conclusion. The change in the enzymic constitution of the mutants is really never so dramatic as might appear from the papers from Shimwell. A further study of the eventual genetic homogeneity of both biotypes by means of mutations, transformations and recombinations is in progress in our laboratory.

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The Effect of Monovalent Cations on the Inhibition of Yeast Metabolism by Nystatin

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SUMMARY

Glycolysis of yeast is inhibited by the polyene antibiotic nystatin. This inhibition was prevented or reversed at or above pH 5·8 (the approximate intracellular pH of yeast) by adding NH_4^+, K^+ or Rb^+ (in order of decreasing effectiveness). These ions do not interfere with the absorption of nystatin by the yeast cell. At pH 4 both glycolysis and respiration are highly sensitive to nystatin but are not protected by the monovalent cations. At pH 7 respiration of intact cells was insensitive; only with yeast protoplasts could respiration be inhibited by nystatin. Temporary protection was obtained with NH_4^+ or K^+.

K^+ and NH_4^+ did not prevent the fungicidal or fungistatic actions of nystatin even under conditions where the protection of glycolysis by these ions was essentially complete. The original yeast cells were not dependent upon added K^+. After a brief treatment with nystatin at pH 7·0, the cells showed an absolute requirement for K^+ or NH_4^+; it was shown that K^+ was rapidly lost by cells following contact with nystatin. It is concluded that nystatin directly damages the cell membrane and thus produces a rapid increase in permeability to small ions. The resulting depletion of cellular K^+ halts glycolysis. The addition of K^+ or NH_4^+ restores glycolysis but does not reverse the membrane alterations.

INTRODUCTION

Nystatin, a polyene antifungal antibiotic produced by *Streptomyces noursei* (Brown & Hazen, 1957), inhibits growth and utilization of various substrates by fungi, especially yeast (Lampen, Morgan & Slocum, 1957; Harman & Masterson, 1957; Meyer-Rohm, Hopff & Lange-Brock, 1957; Peynaud, Lafourcade & Lafon, 1957; Bradley, 1958*a*, *b*). Lampen *et al.* (1957) reported that nystatin was more effective at acid than at neutral pH values in inhibiting glycolysis and respiration of *Saccharomyces cerevisiae*. The increased binding of nystatin by yeast at acid pH was suggested as a possible cause for the greater sensitivity of metabolism at this pH. Evidence is now presented that this differential sensitivity is a function both of pH and of the monovalent cations in the test system.

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METHODS

Organisms. Saccharomyces cerevisiae strain A-B was commercial bakers' yeast obtained from Anheuser Busch, Old Bridge, New Jersey, in the form of a pressed filter cake. The cells were washed four times in water before use.

Saccharomyces cerevisiae strain LK2G12 (obtained from Dr H. Halvorson, University of Wisconsin, Madison) was grown for 16 hr. on a slope of Penassay agar (Difco Laboratories, Inc., 920 Henry St, Detroit, Mich.) containing 1% (w/v) glucose. The cells from one slope were transferred into 100 ml. of Wickerham's medium (Wickerham, 1951) and incubated for 16 hr. at 28° on a rotary shaker. These cultures had an optical density of *c.* 1·0 at 530 mμ when measured with the uninoculated medium as a blank. After washing twice with water, the cells (herein termed stationary-phase cells) were suspended in water at a concentration of 1×10^8/ml., as determined by counts in a haemacytometer (American Optical Co., Buffalo, N.Y.). By using the method described by Cavett (1931), the nitrogen content of 1×10^8 cells (3 mg. dry weight) was found to be *c.* 0·22 mg.

To prepare log-phase cells, 10 ml. of a stationary-phase culture was added to 100 ml. of Wickerham's medium and incubated as above for 4 hr. At this time an optical density of approximately 0·4 had been reached. The cells were washed twice with water and resuspended at a concentration of 1×10^8/ml. Buds were counted as individual cells. It was found that 1×10^8 cells (2 mg. dry wt.) contained *c.* 0·05 mg. N.

Protoplasts were obtained from log-phase cells by following essentially the technique described by Bachmann & Bonner (1959) for *Neurospora crassa.* Before adding the snail enzyme to the cells, 0·1 ml. of a 1% (w/v) aqueous cysteine-HCl solution was added to each ml. of enzyme to overcome the inhibitory effect of thiomersalate, present as a preservative in the commercial enzyme solution. The protoplasts were washed twice with sodium citrate + phosphate buffer (pH 5·8) plus 0·82 M-mannitol, and resuspended in buffered stabilizer solution. The nitrogen content was *c.* 0·075 mg./1×10^8 protoplasts.

Sensitivity tests. Defined medium N of Vogel (1956) was used with the following additions, per l., 10 g. glucose, 2·0 mg. *i*-inositol, 0·2 mg. calcium pantothenate 0·2 mg. pyridoxine.HCl and 0·2 mg. thiamine.HCl. A 50 ml. quantity of medium was inoculated with 0·5 ml. of a stationary-phase culture of strain LK2G12. Nystatin was sterilized by the method of Donovick *et al.* (1955) and added aseptically to the autoclaved medium. The cultures were incubated for 16 hr. at 28° on a rotary shaker. The criterion of growth was the optical density value at 660 mμ.

Manometry. The conventional Warburg respirometer was used at 30°. The contents of the flasks will be described below. Since 0·82 M-mannitol was present as an osmotic stabilizer in the experiments with protoplasts, it was generally added in tests with log-phase cells to permit direct comparison between the two cell types. The sensitivity of the log-phase cells to nystatin was increased somewhat by the addition of mannitol, but the characteristics of the inhibition did not appear to be altered. The sensitivity of stationary-phase cells was not affected by mannitol.

Preparation of materials. A highly purified preparation of nystatin (Lot HV942, obtained from the Squibb Institute for Medical Research, New Brunswick, N.J.) was dissolved in dimethyl sulphoxide and diluted in water. The maximum concentration of dimethyl sulphoxide in any reaction mixture (0·07 %) had no detectable effect on metabolism. Buffers were prepared as described by Gomori (1955). Na^+ was replaced in certain experiments by K^+ or NH_4^+.

RESULTS

Protection of glycolysis by potassium ions

In the experiments previously reported (Lampen *et al.* 1957), the buffers used at neutral pH value contained considerable quantities of both sodium and potassium ions. In subsequent tests with only a single cation present (Table 1), it became evident that glycolysis was highly sensitive to nystatin at pH 7·0 in Na^+-buffer, but was unaffected in K^+-buffer. At pH 4·0 there was no significant protection by K^+. The amount of nystatin absorbed by the cells was the same whether Na^+ or K^+ was present (Table 1); thus K^+ did not protect by preventing the access of nystatin to the yeast cells. More nystatin was absorbed at acid than at neutral pH, but, as can be seen from Fig. 1, the sensitivity of glycolysis to nystatin was not greatly affected. The inhibition did occur more rapidly at pH 4·0 than at pH 7·0; however, the rate in the absence of nystatin was also higher at pH 4·0. Thus, the total CO_2 production in the presence of differing nystatin levels was similar at both pH values.

Table 1. *The effect of* Na^+ *and* K^+ *ions on the inhibition of yeast glycolysis by nystatin at different pH values*

Glycolytic activity is expressed as μl. CO_2 evolved in 60 min./1×10^8 cells. Uptake of nystatin is given as μg./mg. (dry weight) of yeast in 30 min. (Lampen *et al.* 1959). Each vessel contained 9×10^7 stationary-phase cells of *S. cerevisiae* LK2G12 and 2·8 ml. citrate + phosphate buffer, with use of the sodium or potassium salts indicated below. The nystatin and 60 μmole of glucose were added at 0 min. Final volume: 3·0 ml. Gas phase: N_2. Temperature: 30°.

	Glycolysis		Uptake of nystatin
μg. nystatin/ml.	0	5	5
pH 4·0 (Na^+)	572	69	3·0
pH 4·0 (K^+)	595	68	3·0
pH 7·0 (Na^+)	384	89	0·7
pH 7·0 (K^+)	421	359	0·7

Protection by various cations

With the A-B cells or stationary-phase cells of strain LK2G12, essentially complete protection of glycolysis was obtained when either K^+ or NH_4^+ was added to sodium citrate + phosphate buffer (pH7) system. This is illustrated in Fig. 2. At a concentration of 4 mM-KCl or NH_4Cl, good glycolytic activity was usually maintained; in subsequent experiments both were generally added at 9 mM to insure adequate protection. The addition of K^+ or NH_4^+ or both to the control vessels caused a slight stimulation of the rate of glycolysis or respiration at pH 7·0. The rate of increase, however, was a relatively minor effect when compared with the reversal rate of completely inhibited cells after the addition of K^+ or NH_4^+.

NH_4^+ was more effective than K^+ in protecting log-phase cells or the protoplasts derived from them. Thus, with log-phase cells in sodium citrate + phosphate buffer, a rapid inhibition of glycolysis was produced by 0·25 μg. nystatin/ml. The effect of

1 μg./ml. was prevented by NH_4^+ but only partially by K^+. At 10 μg. nystatin/ml., the addition of either ion (or both) yielded only *c.* 40 % of the uninhibited rate of CO_2 production. The superiority of NH_4^+ was equally evident in tests with protoplasts. Glycolysis was rapidly eliminated by 0·25 μg. nystatin/ml.; as shown in Fig. 3, the action of 1 μg./ml. was prevented for at least 60 min. by NH_4^+, and only delayed by K^+.

At pH 7·0 respiration of protoplasts was inhibited only when a nystatin concentration of 20 μg./ml. was attained. Addition of NH_4^+ or K^+ resulted in temporary protection, but after 40 min. respiration slowed abruptly and it was observed that the protoplasts had lysed.

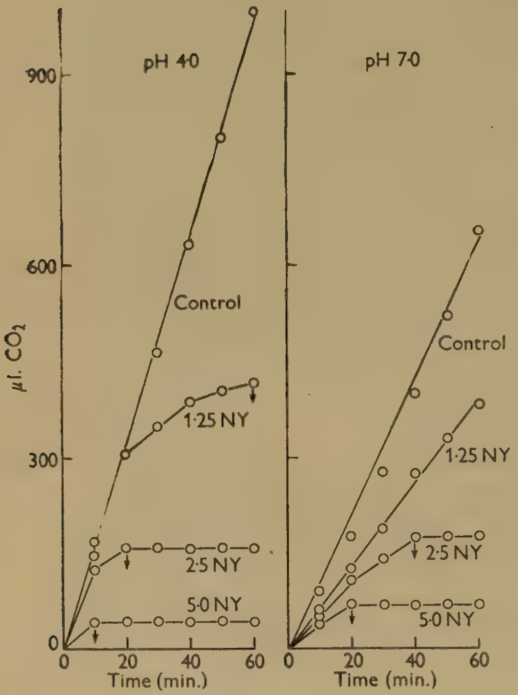

Fig. 1

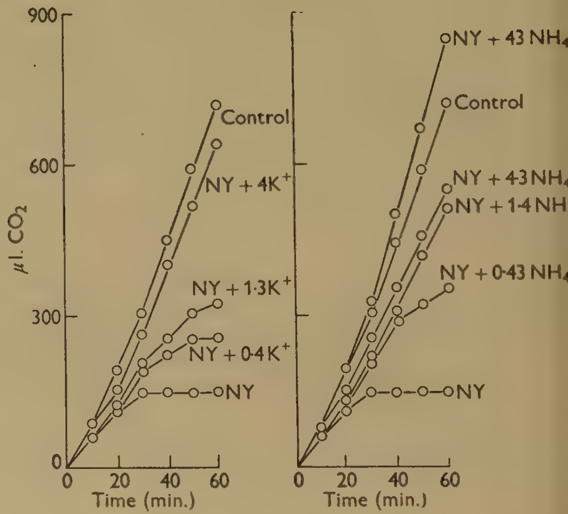

Fig. 2

Fig. 1. Sensitivity of glycolysis to nystatin at neutral and at acid pH. Each vessel contained 2·5 mg. (dry weight) of *S. cerevisiae* strain A-B cells, 1·5 ml. pH 4·0 or 7·0 sodium citrate + phosphate buffer, and water to bring the final volume to 3·0 ml. Nystatin (NY) concentrations are in μg./ml. The nystatin and 60 μ mole glucose were added from the side-arm at 0 min. Gas phase: N_2. Temperature: 30°. The (↓) indicates the point at which metabolism ceased.

Fig. 2. The protection of yeast glycolysis by various concentrations of K^+ or NH_4^+ against inhibition by nystatin. Each vessel contained 2·5 mg. (dry weight) of *S. cerevisiae* strain A-B cells, 1·5 ml. sodium citrate + phosphate buffer (pH 7), K^+ or NH_4^+ in the indicated final concentrations (expressed in m-equiv./l.), and water to 3·0 ml. At 0 min., 120 μmole glucose and, where indicated, 15 μg. nystatin were added from the side-arm. Gas phase: N_2. Temperature: 30°.

Respiration by intact cells, either from log- or stationary-phase cultures, was highly resistant to nystatin at neutral pH. No inhibition was observed in sodium citrate+phosphate buffer upon the addition of 40 μg. nystatin/ml.; at pH 5·8, log-phase cells were inhibited by 20 μg./ml. but stationary-phase cells were only partially inhibited by 40 μg./ml. No protection was achieved by adding a mixture of K^+ and NH_4^+. At pH 4·0 respiration was relatively sensitive to nystatin: A-B cells were inhibited by 2 μg./ml. and log-phase LK2G12 cells by 0·25 μg./ml. No significant protection was afforded by 0·01–0·1 M-KCl and/or 0·01 M-NH_4Cl.

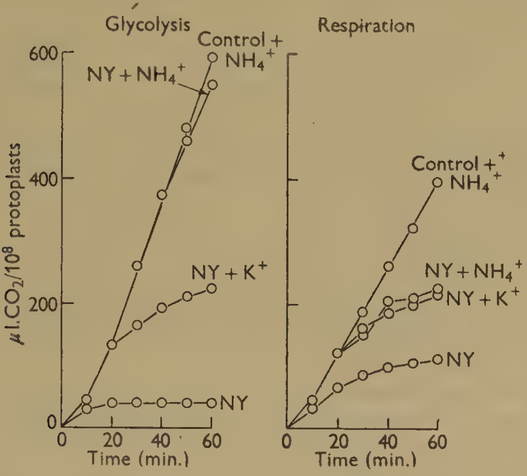

Fig. 3. Protection of glycolysis and respiration in yeast protoplasts. For measurements of glycolysis each vessel contained 8×10^7 protoplasts prepared from *S. cerevisiae* LK2G12, 9 mM-KCl or NH_4Cl (where indicated), 1 μg. nystatin/ml. (where indicated), and a gas phase of N_2; for respiration, $1·6 \times 10^8$ protoplasts, 18 mM-KCl or NH_4Cl, 20 μg. nystatin/ml., and an atmosphere of air were used. All vessels received 2·7 ml. sodium citrate + phosphate buffer (pH 7·0) containing 0·82 M-mannitol and water to a total volume of 3·0 ml. Temperature: 30°. The nystatin and 120 μmole glucose were added at 0 min.

Reversal of the inhibition of glycolysis

In cells inhibited by nystatin at neutral pH, glycolysis could be initiated by adding K^+ or NH_4^+ as long as 1 hr. after total inhibition had occurred. From the experiment presented in Fig. 4A, it may be noted that CO_2 production resumed within 10 min. after addition of the supplement and attained a rate approximately that in control vessels.

Reversal of inhibition could also be obtained (Fig. 4B) by the addition of rubidium salts at relatively high levels (*c.* 50 mM). Lithium chloride and magnesium sulphate were without effect at 9 or 50 mM, and were somewhat toxic at 50 mM in the absence of nystatin. Calcium, ferrous or manganous salts were inactive at 9 mM. At this value none of these ions prevented complete reactivation when a $K^+ + NH_4^+$ mixture was subsequently added.

Since 0·1 or 0·2 M sodium salts were present in most experiments, it was conceivable that the inhibition by nystatin was essentially an induced sodium toxicity. When 0·1 M-imidazole-HCl buffer (pH 7·0) was utilized, the sensitivity of glycolysis to nystatin did not differ significantly from that observed in sodium citrate + phosphate buffer or in unbuffered medium. Thus a high concentration of Na^+ was not essential for the inhibition.

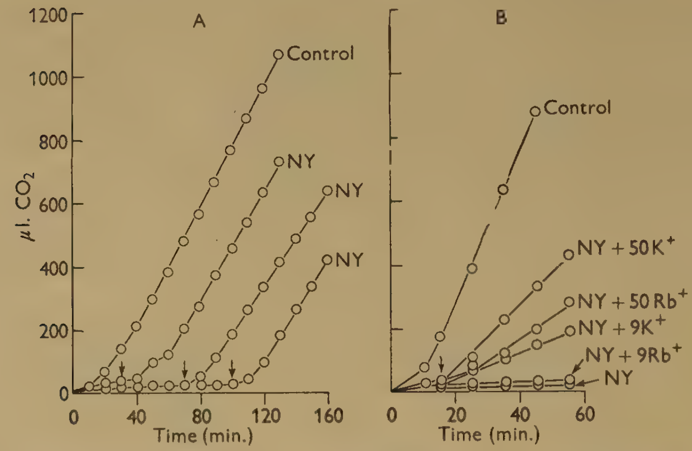

Fig. 4. Reversal by monovalent cations of the inhibition of glycolysis by nystatin. Each vessel contained 1×10^8 cells of *S. cerevisiae* LK2G12 from a log-phase culture and 2·5 ml. sodium citrate + phosphate buffer (pH 7·0), containing 0·82 M-mannitol. The nystatin (3 μg.) and 120 μmole glucose were added from the side-arm at 0 min. Final volume: 3·0 ml. Gas phase: N_2. Temperature: 30°.

A. At 30, 70 or 100 min. as indicated by (↓), KCl and NH_4Cl were tipped into individual vessels to yield final concentrations of 9 mM.

B. At 15 min. (↓) various concentrations of RbCl or KCl were tipped into individual vessels. Concentrations are expressed as m-equiv./l.

The specific anions present do not appear to be critical. Reversal occurred in test mixtures containing either phosphate, citrate or chloride ions, and the addition of phosphate did not affect the rate at which reversal was attained. The concentration of K^+ or NH^+ required for protection was similar whether chloride, nitrate or sulphate salts were used.

Protection or reversal of the inhibition was obtained with a $K^+ + NH_4^+$ mixture only at pH 5·8 and above (Table 2). Conway & Downey (1950) have reported that the intracellular pH of yeast is 5·8–6·2. At a pH below 5·8 an increase in cellular permeability (induced by nystatin) may produce a rapid acidification of the intracellular space and initiate irreversible damage to the glycolytic system. Consistent

Table 2. *Effect of* pH *value on the reversal by* K^+ *and* NH_4^+ *of the inhibition of glycolysis by nystatin*

Each vessel received either 1×10^8 log-phase cells of *S. cerevisiae* LK2G12 or 8×10^7 protoplasts and 2·6 ml. sodium citrate-phosphate buffer containing 0·82 M-mannitol. The nystatin and 120 μmole glucose were added from the side arm at 0 min. Final volume: 3·0 ml. Gas phase: N_2. Temperature: 30°. After 20 min. incubation, glycolysis had ceased in those vessels containing nystatin. KCl and HN_4Cl were then added to all vessels in final concentrations of 9 mM, and gas evolution was measured for an additional 40 min.

		Cells								Protoplasts	
pH		4·0		5·0		5·8		7·0		7·0	
μg. nystatin/ml.		0	1	0	1	0	1	0	1	0	1
μl. CO_2 produced/10^8 cells	0–20 min.	203	11	146	12	104	13	104	31	125	40
	20–60	712	2	514	7	284	122	280	229	335	90

with this suggestion is the observation that cells treated for 15 min. at pH 4·0 with 5 µg. nystatin/ml. and then washed, did not resume glycolysis even when tested in the absence of nystatin in potassium citrate + phosphate buffer (pH 7·0) containing 10 mM-NH_4Cl.

The differential effect of pH on protection by K^+ is not a function of the conditions under which the original contact between nystatin and the cells occurs. Cells incubated with nystatin (5 µg./ml.) at pH 7·0 in potassium citrate + phosphate buffer and then washed free of unabsorbed nystatin were inactive when tested at pH 4·0 in K^+-buffer; good glycolytic activity was retained at pH 7·0 provided K^+-buffer was used.

With protoplasts, reversal by K^+ and NH_4^+ of an established inhibition was incomplete even at pH 7·0 (Table 2), although excellent protection by NH_4^+ was obtained (Fig. 3). The changes produced by nystatin may result in more severe damage to the protoplast membrane than to the membrane of intact cells which have a protective rigid cell wall. In fact, with protoplasts lack of reversal by K^+-NH_4^+ appeared to correlate with lysis of the preparations.

Effects of nystatin not prevented by K^+ *or* NH_4^+

Despite the fact that K^+ and NH_4^+ protect glycolysis against concentrations of nystatin rapidly inhibitory in sodium citrate + phosphate buffers, it is clear from several lines of evidence that these ions do not protect the cells completely from the action of nystatin. High concentrations of nystatin are inhibitory to glycolysis even in the presence of excess K^+ and NH_4^+. The mechanism by which this inhibition is produced will be considered in a subsequent publication.

With low concentrations of nystatin (5 µg. or less/ml.), the addition of K^+ and NH_4^+ afforded complete protection of glycolysis against the agent but did not prevent its fungicidal action. As is illustrated in Table 3, a decrease of *c.* 99·99 % in colony-forming units occurred regardless of the presence or absence of the ions.

Table 3. *Lack of effect of* NH_4^+ *and* K^+ *on the fungicidal action of nystatin*

The manometric measurements are expressed as µl. CO_2 evolved in 40 min. Each vessel contained $2·8 \times 10^8$ cells of *S. cerevisiae* LK2G12, harvested from a stationary-phase culture, and 1·5 ml. sodium citrate + phosphate buffer (pH 7·0). KCl and NH_4Cl were added where indicated to yield final concentrations of 9 mM. The nystatin and 120 µmole glucose were added from the side-arm at 0 min. Final volume: 3·0 ml. Gas phase: N_2. Temperature: 30°. After 40 min. the cells were diluted in potassium citrate + phosphate buffer (pH 7·0) and plated on Wickerham's medium adjusted to pH 7·2 and containing 1·5 % agar. Colonies were counted after incubation of the plates for 48 hr. at 28°.

µg. nystatin/ml.	Addition	CO_2	Colony count/ml.
None	None	338	$9·2 \times 10^7$
5	None	80	$7·5 \times 10^3$
None	$NH_4Cl + KCl$	346	$6·6 \times 10^7$
5	$NH_4Cl + KCl$	348	$7·5 \times 10^2$

Evidence that nystatin initiates a rapid alteration in cellular permeability is presented in Table 4. Glycolysis by the original cells was essentially independent of exogenous K^+ (Table 1). Even with cells grown on the defined medium modified to contain growth-limiting amounts of potassium salts there was only a slight stimulation of glycolysis when 9 mM-KCl was added to the usual sodium citrate + phosphate buffer (pH 7·0). In contrast, when cells were incubated with 5 μg. nystatin/ml. in potassium citrate + phosphate buffer (pH 7·0) for 30 min., washed quickly with water, and their glycolytic activities again measured, these cells now had an absolute requirement for exogenous K^+ (Table 4). Respiration, insensitive to nystatin at pH 7·0 by direct test, was also totally dependent on added K^+. These nystatin-treated cells could also glycolyse or respire actively when resuspended in ammonium citrate-phosphate buffer (pH 7·0); in fact, the rates were higher in the presence of NH_4^+ than of K^+.

Table 4. *Requirement of* K^+ *or* NH_4^+ *for glycolysis and respiration after nystatin treatment*

Treatment (1) $1\cdot4 \times 10^8$ cells of *S. cerevisiae* LK2G12 from a stationary-phase culture were suspended in 3·0 ml. of the K^+ citrate + phosphate buffer (pH 7·0, 175 m-equiv. K^+/l.) in the presence of 60 μmole glucose and, where indicated, 5 μg. nystatin/ml. The test mixture shaken 40 min. at 30° in an atmosphere of N_2.

Treatment (2) The cell samples were washed twice with water and added to Warburg flasks containing 1·5 ml. sodium, potassium, or ammonium citrate-phosphate buffer (pH 7·0) in a final volume of 3·0 ml. At 0 min. 60 μmole glucose was added from the side-arm. Gas phase: N_2 or air. Temperature: 30°.

	μg. nystatin/ml. during treatment (1)			
Buffer	0	5	0	5
Treatment (2)	μl. CO_2/60 min.		μl. O_2/60 min.	
Na^+	440	12	342	11
K^+	850	525	330	230
NH_4^+	880	695	338	306

The growth of yeast is highly sensitive to nystatin at pH 7·0, even in the presence of high concentrations of K^+ and NH_4^+. For instance, the minimal inhibitory concentration for strain LK2G12 was *c.* 0·5 μg. nystatin/ml. in either the crude Wickerham medium (20 m-equiv. K^+/l.) or Vogel's defined medium (36 m-equiv. K^+/l.). If the potassium salts in the latter medium were replaced by sodium salts so that

Table 5. *Leakage of* K^+ *from yeast cells following treatment with nystatin*

The test mixtures contained 5×10^8 cells of *S. cerevisiae* LK2G12, 15 ml. sodium or ammonium citrate + phosphate buffer (pH 7·0), 1200 μmole glucose, nystatin where indicated, and water to 30 ml. After incubation for 30 min. at 30° in an atmosphere of N_2, the cells were collected by centrifugation, washed once with water, and resuspended in 5 ml. of water. The suspensions were placed in a boiling water bath for 3 min., the cellular debris was removed by centrifugation, and the supernatant fluid analysed for potassium. Conventional flame photometric techniques were employed in conjunction with a Beckman spectrophotometer, model B.

		μg. K^+/mg. cells (dry wt.)	
μg. nystatin/ml.	Buffer cation	Stationary-phase cells	Log-phase cells
0	(H_2O)	14	8
0	Na^+	9	10
5	Na^+	0·8	1·1
5	NH_4^+	1·0	—

only a trace of K^+ was present, the extent and rate of growth were decreased substantially. Nevertheless, under these conditions the concentration of nystatin required to inhibit growth was still one-half that observed with ample K^+. The sensitivity of growth was unchanged when, in K^+-deficient medium, NH_4^+ was replaced by 1 % Casamino acids (Difco). Thus K^+ and NH_4^+ are not decisive in determining the sensitivity of growth to nystatin.

Leakage of K^+ *from cells*

Incubation of log- or stationary-phase yeast cells with nystatin produced a rapid decrease in the intracellular concentration of K^+. This is illustrated by the experiment (Table 5) in which bound K^+ (not readily removed by washing with water) was measured. The efflux of K^+ was not prevented by levels of NH_4^+ which completely protected glycolytic activity. Thus NH_4^+ did not prevent the damage to the cell membrane by nystatin.

DISCUSSION

Previous studies on the inhibition of yeast by nystatin were conducted primarily at acid pH. These experiments focused attention on an irreversible inhibition of glycolytic and respiratory activity as a critical factor in the fungicidal action of nystatin. Evidence is now presented that the metabolic changes are secondary to alterations of the cell membrane. These alterations are themselves apparently irreversible, but can, at appropriate pH values, produce either a reversible inhibition of glycolysis or the destruction of the enzymes.

The most attractive working hypothesis is that nystatin reacts primarily with the cell membrane. Nystatin can be bound by isolated cell walls (Lampen & Arnow, unpublished observations), but the critical importance of the membrane is clear since the sensitivity of protoplasts to nystatin is essentially identical with that of the log-phase cells from which they were derived (Sutton, Marini & Lampen, 1960). The binding of nystatin causes the membrane to become highly permeable to K^+ and perhaps to other monovalent cations, and glycolysis quickly ceases as a result of the deficiency of K^+. At neutral pH, and when the concentration of nystatin does not exceed 10–20μg./ml., the addition of K^+ or NH_4^+ will permit glycolysis to resume. There is no evidence for destruction of the glycolytic enzymes under these conditions since essentially complete reactivation with the monovalent cations is possible. The essentiality of K^+ or NH_4^+ for glycolysis has long been recognized. These ions are required for yeast phosphofructokinase (Muntz, 1947), aldolase (Richards & Rutter, 1960) and pyruvate kinase (Washio, Mano & Shimazono, 1959). NH_4^+ is more effective than K^+ in reactivating phosphofructokinase; this difference may in part account for the superiority of NH_4^+ for nystatin-treated cells. The loss of cellular K^+ which occurs in the presence of nystatin does not halt respiration unless the cells are subsequently washed. Since complete reactivation by K^+ and NH_4^+ can be achieved, an increased membrane permeability appears to be an adequate explanation of the observed inhibition.

The initial interaction of nystatin and the cell membrane at pH 4 may well be identical with that obtained at pH 7, since the concentration of nystatin required to inhibit glycolysis or respiration at pH 4 approximates that needed to inhibit glycolysis at pH 7 (in the absence of K^+ or NH_4^+). At reactions more acid than the usual intracellular pH value of yeast, sudden increase in the permeability of the cell membrane to monovalent cations would produce a rapid acidification of the cell interior. This may activate latent lytic enzymes, as occurs following acidification of animal cells (De Duve, 1957), and thus initiate the decrease in extractable glycolytic enzymes which characterizes the action of nystatin at pH 4·5 (Scholz, Schmitz, Bücher & Lampen, 1959). It should be noted that the inhibition of glycolysis and respiration is clearly a result of the prior increase in membrane permeability. This change may be the primary event in nystatin action, since an irreversible loss of ability to maintain elevated internal concentrations of critical ions and cofactors would certainly be lethal.

Evidence has been reported by several laboratories that nystatin produces an alteration of cellular permeability with subsequent leakage of internal constituents. A critical role for K^+ and NH_4^+ has not been previously proposed, however. Osteux, Tran-Van-Ky & Biquet (1958) noted that the rate of excretion by *Candida albicans* of the acids of the citric acid cycle and of inorganic phosphate was increased in the presence of nystatin. The leakage of phosphate was correlated with the concentration of nystatin and the time of contact between the cells and the antibiotic. Bradley (1958*a*) observed a decrease in cellular phosphorus during a 3 hr. period following the addition of nystatin, but at shorter intervals actual accumulation of phosphate esters was noted by Scholz *et al.* (1959), while pyruvate and malate were rapidly released from the cell. Under the conditions of the present experiments, low concentrations of nystatin appear first to cause a deficiency of K^+ and NH_4^+. With higher concentrations of nystatin, leakage of various low molecular weight substances probably occurs, since restoration of activity requires the addition of all the cofactors needed in the usual soluble glycolytic system (Lampen & Arnow, unpublished observations).

Bradley & Farber (1960) observed that nystatin inhibited fermentation by *Candida stellatoidea* in various buffers only at relatively acid pH (pH 2–5). These workers emphasized the potential role of the anions in determining sensitivity, but the cations employed were not reported. Ribéreau-Gayon, Peynaud, Lafourcade & Lafon (1958) also reported that nystatin more effectively inhibited glycolysis of *Saccharomyces cerevisiae* at acid than at alkaline pH, but again these authors did not indicate the cations used in their buffer systems.

Although major attention has centred on the disorganization of cell-wall synthesis by antibacterial antibiotics (Salton, 1960), several agents are believed to affect primarily the cell membrane and hence cellular permeability. Newton (1956) demonstrated this for polymyxin; Anand & Davis (1960) and Anand, Davis & Armitage (1960) reported similar results for streptomycin. Gale (1955) offered evidence that β-propiolactone acts on the cell membrane of yeast. Scharf (1960) has recently observed K^+ leakage following treatment of yeast with benzalkonium chloride. Nystatin appears to be an excellent example of this type of antibiotic.

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Morphological and Biological Properties of a Strain of Chromogenic Acid-fast Bacteria and of its Phage-immune Variant

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SUMMARY

Phage-immune variants were detected among chromogenic acid-fast mycobacteria by the action of *Mycobacterium phlei* bacteriophage. One of the strains thus obtained showed not only phage immunity, but also a marked difference in colonial appearance, pigmentation, antigenic composition and pathogenicity for laboratory animals as compared with the original strain.

INTRODUCTION

During a recent survey of the phage susceptibility of chromogenic acid-fast bacteria isolated from patients with tuberculous disease, it was found that twenty-two out of ninety-eight of these strains were affected by exposure to one or more of the six phages used. In nine instances phage-immune dissociates were isolated from hazy plaques or from isolated colonies within clear plaques. Several of the phage-immune bacteria showed some morphological differences from the parent strain. In one case, the differences were so marked that this strain became the subject of the present study.

METHODS

Source of the strain of Mycobacterium. The chromogenic strain of acid-fast bacterium 41308 was isolated in 1956 from the sputum of a patient who, 26 months before, presented radiological evidence of widespread infiltration with bilateral cavitation. Typical *Mycobacterium tuberculosis* was isolated from the sputum at that time and twice again during the first 2 months of treatment. The patient received streptomycin, isonicotinic acid hydrazide and *p*-aminosalicylic acid for 2 years. Because the tuberculous disease appeared to be 'arrested', chemotherapy was suspended. Two months later, pure cultures of chromogenic acid-fast bacteria were recovered from the sputum.

Source of bacteriophages. The phages used were derived from *Mycobacterium phlei*, *M. friburgensis*, *M. rabinowitsch*, *M. smegmatis* and were received from Dr E. Vandra, Budapest, Hungary, and also phages D28 and D29 received from Dr S. Froman, Olive View, California, U.S.A.

Characters of the chromogenic acid-fast bacterium and phage-immune variant studied: (*a*) cultural requirements and colonial appearance; (*b*) microscopic examination of the bacterial elements; (*c*) comparison of their antigenic compositions by agar diffusion precipitation tests; (*d*) susceptibility to antituberculosis agents; (*e*) virulence tested by the neutral red cytochemical reaction and animal inoculation.

RESULTS

Strain 41308 produced smooth light-yellow colonies on egg-yolk media after incubation periods of 10–12 days at 37°, and of 16–18 days at 22° (Pl. 1, fig. 1). The pigment was not affected by light, and the strain was thought to belong to group III of Runyon's classification (Runyon, 1959). According to agar diffusion precipitation studies (Mankiewicz, 1958) this strain belonged to a group of about 6% of acid-fast bacteria which show a close antigenic relationship with virulent tubercle bacilli. When this strain was spot tested with *Mycobacterium phlei* phage according to the technique described by Froman & Bogen (1954) and modified by Takeya, Yoshimura, Tamamura & Toda (1959), a slightly hazy plaque of regular contour was obtained. The subcultures of the phage-immune bacteria showed smooth colonies of darker pigmentation than those of the parent strain. Both strains grew only on media designed for *M. tuberculosis*.

When 0·2 ml. of the *Mycobacterium phlei* phage filtrate (referred to as phage M) containing 10^8 phage particles/ml. were added to 4·8 ml. of a 48-hr. culture of strain 41308 in Tween albumin medium, subcultures made during the first 2 weeks on egg-yolk medium yielded either the original light-yellow colonies, or the more darkly pigmented colonies described above. After 2 weeks fewer colonies of a different aspect appeared on subculture. These colonies of a phage-immune variant were umbilicated or wart-like and at 37° their pigment was orange. They lowered the pH value of the medium containing malachite green (Pl. 1, fig. 2). These growth characteristics were maintained through fifteen subcultures on egg-yolk medium at 37°. By maintaining the above-described conditions of incubation with phage M, the phage-immune variant was isolated on several occasions from single colonies of strain 41308 which were selected after repeated plating and inoculated into liquid Tween albumin medium.

Besides the difference in the colonial aspect, the phage-immune variant grew on egg-yolk medium in 3 days at 37°, and in 5 days at 22°. It grew readily on nutrient agar and in brain-heart infusion. On agar medium, the colonies resembled those of Nocardia: they were lighter in colour than those grown on egg-yolk media, and were dry and granular. In brain-heart infusion, the variant produced a granular deposit at the bottom of the tube, and a veil at the surface of the liquid. Like certain *Nocardia* spp., it was able to use paraffin as sole carbon source. In contrast to the parent strain, the pigment of the variant was affected by light. At room temperature and in the light, only a light pigment was formed; the colonies were small, granular and closely resembled those of *Mycobacterium tuberculosis* (Pl. 1, fig. 3).

On microscopic examination, strain 41308 showed slender granulated rods. Approximately 80% of the bacilli of a 10-day culture in Tween albumin medium were acid-fast. Under the same conditions only 10% of the slender granulated phage-immune bacteria were acid-fast. There were no differences in the susceptibilities of the two strains to streptomycin or isonicotinic acid hydrazide (INH); both strains were susceptible to 1 μg. streptomycin/ml. and resistant to 10 μg. INH/ml. However, there was a significant difference in their susceptibility to ethionamide

(TH 1314), the original strain being sensitive to 0·5 μg./ml. while the phage-resistant strain was resistant to 10 μg./ml. of this antituberculosis drug.

The most remarkable difference between strain 41308 and its variant was that the latter was lysogenic. Culture filtrates of the phage-immune strain lysed the parent strain 41308. In order to eliminate the possibility that the lysis was due to adsorbed phage, single colonies of the variant were transferred five times on egg-yolk media. One colony of the last culture was selected as inoculum for Dubos broth basal medium containing Tween 80, which is known to interfere, in the absence of albumin, with the adsorption of phages to bacteria (White & Knight, 1958). Filtrates from the Dubos broth basal culture lysed the same mycobacteria as did phage M, namely, *Mycobacterium phlei*, *M. stercoides*, *M. friburgensis*, strain 41308 and five out of twelve test strains of unnamed chromogenic bacteria. The lytic spectrum suggested that the lysogenic variant carries a prophage similar to, or identical with phage M.

Froman *et al.* (1955) reported that BCG strains did not show as high and as consistent a susceptibility to mycobacteriophages as did strains H37Rv and H37Ra. Takeya *et al.* (1959) found that attenuated strains of human tubercle bacilli of Imamura were less susceptible to phage action than the original virulent strains. The attenuated strains, especially those of Imamura, showed changes in colonial appearance which were similar to those undergone by the variant of 41308. A study of the antigenicity and pathogenicity of this variant was attempted as follows.

(*a*) The agar diffusion precipitation technique (Mankiewicz, 1958) was used to study the antigenic composition of the variant. It was found that the precipitation pattern had changed from that of the original strain. The variant shared antigens with *Mycobacterium phlei*, with *Nocardia asteroides* and with other chromogenic acid-fast bacteria to a much larger extent than with virulent tubercle bacilli.

(*b*) The cytochemical reaction with neutral red (Dubos & Middlebrook, 1948) was used with the following results:

Strain 41308	+
Its phage-immune variant	–
Controls	
H37Rv	++
BCG	(±)

(*c*) Animal experiments. Five guinea-pigs (250 g.) were inoculated intramuscularly with 1 mg. wet weight of strain 41308, and five others with the same weight of the variant. After 4 and 8 weeks, the animals inoculated with 41308 gave strongly positive reactions to intradermal Old Tuberculin, whereas the guinea-pigs inoculated with the variant remained tuberculin-negative throughout the experiment. After 9 weeks, the latter did not show any sign of disease at autopsy. The animals inoculated with the original strain showed enlarged regional lymph nodes. However, liver and lungs were free from disease and no acid-fast bacteria were isolated from these tissues.

Forty Swiss mice (Rockefeller strain; 20 g.) were inoculated intravenously with 0·5 mg. each of strain 41308; and forty others with the same amount of the variant organism. The animals were killed after 6 weeks. Thirty-two of the mice inoculated with the original strain showed macroscopic lesions in the lungs. None was observed in the animals inoculated with the variant strain. Homogenates of the right lungs and of the right kidneys of the eighty animals were inoculated on egg-yolk medium. Chromogenic, smooth-growing acid-fast bacteria were recovered from cultures of the kidneys (twelve instances) and from the lungs (twenty-six instances) from animals inoculated with strain 41308. No bacteria were recovered from the forty kidney and forty lung cultures of the mice inoculated with the variant (Table 1).

Table 1. *Comparison between the chromogenic acid-fast bacterium* 41308 *and its phage-immune variant*

	Strain 41308	Variant strain
Growth on egg-yolk medium		
At 37° in the dark:	Yellow, smooth,	Orange, umbilicated,
Matures in:	10–12 days	3 days
At 22° in the light:	Yellow, smooth,	Light yellow, granular,
Matures in:	16–18 days	3 days
Growth on nutrient agar:	No growth	Light yellow, granular,
Matures in:		5 days
Susceptibility to ethionamide:	Sensitive	Resistant
Neutral red reaction:	+	−
Sensitization of guinea-pigs to Old Tuberculin:	+	−
Virulence for mice:	+	−
Lysogenicity:	−	+
Susceptibility to		
Phage M:	+	−
Culture filtrate of variant:	+	−

DISCUSSION

The questions which arise concern the origin and nature of the variant. An attempt may be made to narrow down the possibilities which appear to be as follows:

(1) Culture 41308 when isolated from the patient was 'mixed', containing phage-immune and phage-susceptible bacteria of different morphology.

(2) The phage-immune variant is the result of a spontaneous mutation. In both instances, phage M would have acted as a selective agent for the dissociate.

(3) Strain 41308 is an undetected or defective lysogenic strain.

(4) The change was phage-induced by (*a*) the process of transduction, or (*b*) by lysogenization.

The possibilities that culture 41308 was mixed, or that the variant resulted from spontaneous mutation, can be disregarded because extensive plating of the original strain on egg-yolk medium never produced the faster growing umbilicated variant. Without previous exposure to phage M, no growth was produced by 41308 on nutrient agar, or in Youman's medium with serum containing 10 μg. ethionamide/ml. Strain 41308 did not produce any lysis of strains selected at random. No lysis occurred within this strain after exposure to ultra-violet irradiation. It did not show any immunity to the lytic action of culture filtrates of the variant.

The changes described as characteristic for the variant were induced by phage M only. To establish that the process at work was that of transduction, one would have to know the donor bacterium on which the phage had multiplied. The properties acquired by the variant of 41308 (umbilicated colonial morphology, the particular pigment, resistance to ethionamide, lysogenicity) do not permit tying the variant to

Mycobacterium phlei, *M. stercoides*, *M. friburgensis* or to any of the acid-fast bacteria which were found to be susceptible to phage M. Although the possibility of transduction cannot be completely excluded, lysogenization appears the most plausible mechanism for the production of the variant.

The ability to produce phage and immunity are the most important changes due to lysogenization. Others have been described which concern the morphology and antigenicity of lysogenic bacteria. Ionesco (1953) observed that, due to the presence of prophage, the normally smooth colonies of *Bacillus megaterium* became rough and wart-like. Antigenic changes were described by Uetake, Nakagawa & Akiba (1955), Uetake, Luria & Burrows (1958), Staub, Tinelli, Luderitz & Westphal (1959), Stocker (1958) and Zinder (1957) in Salmonellae after they became hosts to prophages. In Mycobacterium no naturally occurring lysogenic strains have been detected so far. Takeya *et al.* (1959) found that a phage-immune mutant of *Mycobacterium* sp. ATCC 607, resistant to phages A-2 and A-4, was lysogenic.

A connexion between the chromogenicity of colonies of mycobacteria and their immunity to phagolysis has been shown for *Mycobacterium smegmatis* and *M. phlei* (Bassermann, 1959); the phage-immune mutants showed an increase in pigmentation of their colonies.

While extensive studies have been devoted to the localization and chemical nature of the phage receptor groups of phage-susceptible *Escherichia coli* and various salmonellas, little is known about phage receptors in mycobacteria. Penso (1955) suggested that in mycobacteria there exists a close relationship between antigen-antibody reaction and phage susceptibility. Tuberculo-antigens and phage receptors might be of similar molecular structure. The variant of strain 41308 showed that the loss of phage-susceptibility was linked with changes in the antigenic composition as demonstrated by the precipitation studies and the animal experiments. It is hoped that future studies will lead to a definition of the phage-affected areas in mycobacteria.

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EXPLANATION OF PLATE

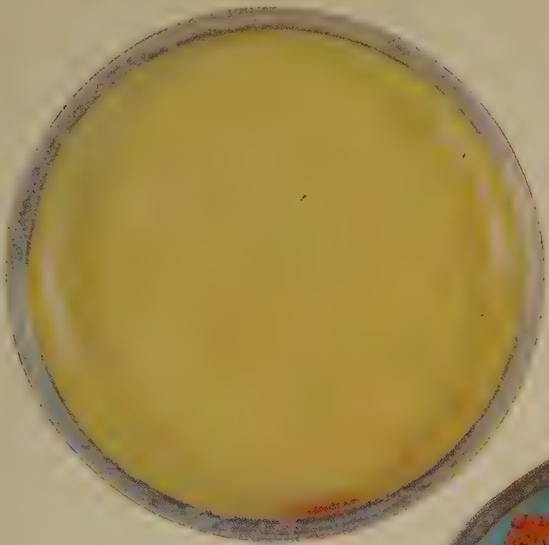
The phage susceptible strain of chromogenic-acid fast bacteria and the phage-immune variant.

Fig. 1. Colonies of the phage-susceptible strain after 12 days of incubation at 37°.

Fig. 2. Colonies of the phage-immune variant after 5 days of incubation at 37° in the dark.

Fig. 3. Colonies of the phage-immune variant after 5 days of incubation at 22° in the light.

1



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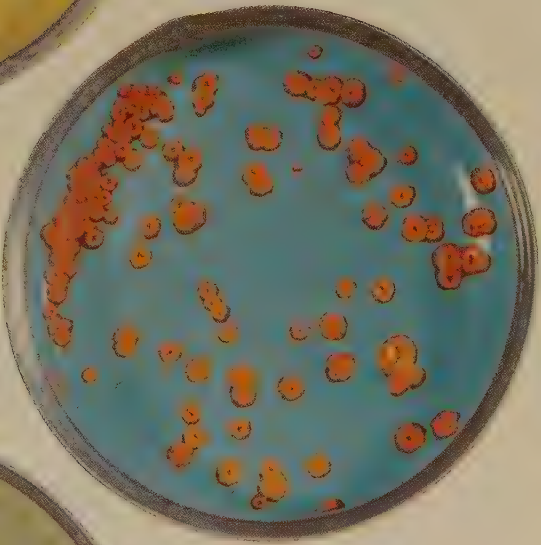

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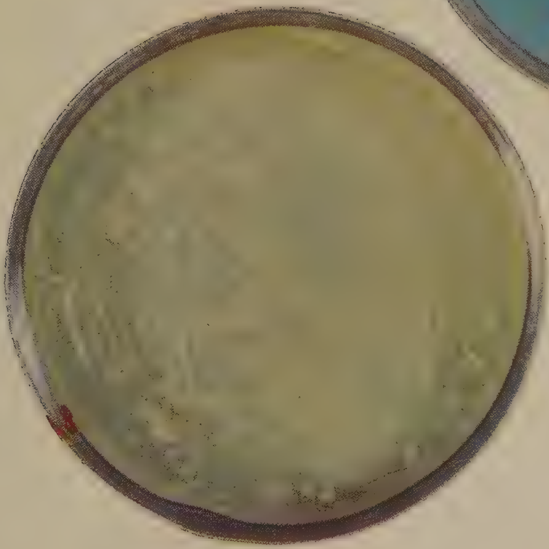

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Effect of Biotin Deficiency on the Synthesis of Nucleic Acids and Protein by *Saccharomyces cerevisiae*

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SUMMARY

A strain of *Saccharomyces cerevisiae*, grown in a medium containing a suboptimal concentration ($0{\cdot}4 \times 10^{-10}$ M) of biotin, was shown to contain less deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and protein but, during the early stages of growth, increased concentrations of acid-soluble ultraviolet (u.v.)-absorbing substances, as compared with the same organism grown in the presence of an optimal concentration ($8{\cdot}0 \times 10^{-10}$ M) of biotin. The concentration of acid-soluble u.v.-absorbing substances in the biotin-deficient yeast was higher, irrespective of the nature of the extracting acid (0·2 N-perchloric acid, 5 % (w/v) and 10 % (w/v) trichloroacetic acid, or 5 % (v/v) *n*-butanol in M/15 KH_2PO_4). Raising the temperature of extraction from 3° to 21° or 30° had little or no effect on the amounts of these u.v.-absorbing substances extracted. Analyses of the nucleotides and nucleobases in the yeast RNA showed these to have a ratio of purine:pyrimidine bases of 1·00–1·15, with the exception of the RNA from 5-day cultures of biotin-deficient yeast which had a slightly but consistently higher ratio. The significance of these results is discussed in relation to the metabolic function of biotin.

INTRODUCTION

Biotin has for some time been recognized as a growth factor for micro-organisms, but, although the metabolic roles of many other vitamins and growth factors have been elucidated, no specific function for biotin in the metabolism of micro-organisms has as yet been unequivocally established. Two main lines of study have been pursued in an attempt to gain information on the role of biotin in microbial metabolism. Growth of certain biotin-requiring micro-organisms in media containing suboptimal concentrations of biotin has been shown to be accompanied by the appearance in the medium of biosynthetic intermediates, the further metabolism of which is inhibited under conditions of biotin deficiency. Accumulation of an aromatic amine, 5-amino-imidazole riboside (Chamberlain, Cutts & Rainbow, 1952; Chamberlain & Rainbow, 1954; Moat, Wilkins & Friedman, 1956) and of hypoxanthine (Chamberlain & Rainbow, 1954) by *Saccharomyces cerevisiae* is the result of an impairment in the ability of the biotin-deficient yeast to complete the synthesis of purines. The inability to synthesize adequate quantities of purine under these conditions is also thought to cause a derangement in the synthesis of pyridine nucleotides by *S. cerevisiae*, which is manifested in the accumulation of nicotinic acid and nicotinic acid adenine dinucleotide in the culture medium (Rose, 1960*a*, *b*), while the same metabolic disturbance is probably responsible for the subnormal contents of adenosine triphosphate (ATP) and coenzyme A in biotin-deficient *Piricularia oryzae* (Katsuki, 1959*a*) and *Bacillus macerans* (Katsuki, 1959*b*).

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Several workers have searched for a more specific locus of biotin function and have been able to show that, in certain biotin-deficient micro-organisms, the activity of some enzymes is markedly depressed. For example, a deficiency of biotin affects adversely the decarboxylation of oxaloacetate in *Lactobacillus arabinosus* (Lardy, Potter & Elvehjem, 1947), oxalosuccinate carboxylation in *Escherichia coli* (Shive & Rogers, 1947) and succinate decarboxylation in *Propionibacterium pentosaceum* (Delwiche, 1950). Biotin has also been shown to be essential for the activity of certain enzymes involved in amino acid metabolism, including those responsible for the aspartate to α-ketoglutarate transformation (Rossi, Rossi & Rossi, 1957), and for the deamination of aspartic acid (Lichstein & Umbreit, 1947), of threonine (Lichstein & Christman, 1948) and of serine (Nadkarni & Sreenivasan, 1957). Hexokinase activity in *Saccharomyces cerevisiae* (Strauss & Moat, 1958) and the ability to convert ornithine and carbamyl phosphate into citrulline in *Streptococcus lactis* (Estes, Ravel & Shive, 1956) have also been reported to be decreased under conditions of biotin deficiency.

However, the function of biotin in these biotin-dependent enzyme reactions has not yet been established. The activity of some of the enzymes in cell-free preparations is known to be stimulated on adding biotin, but a coenzymic role for the vitamin has not been demonstrated directly. Indirect evidence, such as the demonstration of a correlation between the activity of enzyme preparations and the content of bound biotin, is taken by some workers (e.g. Lichstein, 1955) to indicate a possible coenzymic role. There is, however, no evidence to show that the bound forms of biotin which have been isolated (such as ϵ-N-biotinyl-L-lysine, 'biocytin') are coenzymic forms of the vitamin (Wright *et al.* 1952). With other biotin-dependent enzyme systems no stimulation of activity occurs on adding biotin to the cell-free preparations, and this has led some workers to ascribe an indirect role for biotin, probably in enzyme synthesis. Sund, Ravel & Shive (1958), for example, did not obtain any immediate increase in activity of a preparation of the ornithine→citrulline enzyme from biotin-deficient *Streptococcus lactis* on adding biotin, and concluded that biotin is probably concerned in synthesis of the enzyme. Further evidence to support the view that biotin is concerned only indirectly in the activity of this enzyme comes from more recent data (Ravel, Grona, Humphreys & Shive, 1959), which showed that purified preparations of the enzyme contained less biotin than did the original cell-free extract. Similarly, Chambers & Delwiche (1954), as a result of their studies on the function of biotin in *Propionibacterium pentosaceum*, suggested that the vitamin functions in the synthesis of the coenzyme or apoenzyme concerned in the carboxylation of succinate.

The results from both of these lines of study suggest, therefore, that biotin is concerned in protein synthesis, either via the synthesis of purines or in the formation of specific enzymes. The work reported in this paper was carried out in order to examine the effect of biotin deficiency on the synthesis of nucleic acids and total protein in *Saccharomyces cerevisiae*. The results show that, under these conditions, synthesis of both of these groups of substances was impaired.

METHODS

Organism. The strain of *Saccharomyces cerevisiae* (Fleischmann) used was obtained from the Division of Applied Biology, National Research Council of Canada, Ottawa, and was maintained on slopes of malt wort agar: 10 % (w/v) spray-dried malt extract ('Muntona', Munton & Fison Ltd., Stowmarket, Suffolk) +2 % (w/v) agar. Cultures were stored at 3°.

Experimental cultures. The chemically defined medium of Rose & Nickerson (1956) was used. Portions of the medium (100 ml.), containing either an optimal ($8{\cdot}0 \times 10^{-10}$ M) or a suboptimal ($0{\cdot}4 \times 10^{-10}$ M) concentration of biotin, were dispensed into 350 ml. conical flasks, which were plugged and sterilized by autoclaving momentarily at 10 lb./sq.in. The medium was inoculated by the procedure described by Rose (1960*b*), and cultures were incubated statically at 25°. Growth was measured turbidimetrically by determining the optical density of a portion (6 ml.) of culture in the Hilger 'Spekker' absorptiometer (model H 760), using neutral green-grey H 508 filters and a water blank. Optical density measurements were related to dry weight of yeast by a calibration curve.

Nucleic acid estimations. Yeast grown in media containing either an optimal or a suboptimal concentration of biotin was washed three times with M/15 KH_2PO_4, (pH 4·5), and triplicate 3 mg. portions of the crop were taken for nucleic acid estimations. The pellet of yeast was extracted twice, in 15 ml. tapered centrifuge tubes, with 2·0 ml. portions of 0·2 N-perchloric acid at room temperature to remove acid-soluble ultraviolet (u.v.)-absorbing substances. The extracts were pooled, neutralized with N-NaOH, and made up to 5·0 ml. with M/15 KH_2PO_4; the optical density of this extract was measured at 260 mμ, with the Unicam S.P. 500 quartz spectrophotometer, and the reading taken as a measure of the acid-soluble u.v.-absorbing substances in the yeast. The yeast pellet was then extracted twice with 3 ml. of a boiling mixture of 95 % (v/v) ethanol in water (3 vol.)+ether (1 vol.) for 2 min. to extract lipids, and the extracts rejected. The ribonucleic acid (RNA) in the residue was hydrolysed to acid-soluble nucleotides by suspending the material in 2·0 ml. N-NaOH for 1 hr. at room temperature (Schmidt & Thannhauser, 1945; Bonar & Duggan, 1955), after which perchloric acid (N) was added to a concentration of 0·2 N. The supernatant fluid containing the soluble RNA nucleotides was separated from the precipitate of deoxyribonucleic acid (DNA) and protein, which was then washed twice with 1·0 ml. portions of 0·2 N perchloric acid, and the washings combined with the RNA extract. The combined volume was neutralized with N-NaOH, made to 10·0 ml. with M/15 KH_2PO_4, and the optical density at 260 mμ taken as a measure of the RNA content of the yeast.

The residue of DNA and protein was suspended in 2·0 ml. N-perchloric acid, and held at 90° for 15 min. This hydrolysed the DNA to acid-soluble nucleotides, which were removed in the supernatant fluid. Extracts were made up to 3·0 ml. with N-perchloric acid, and the optical density of the solution at 260 mμ taken as a measure of the DNA content of the yeast.

Protein estimations. Protein in the residue remaining after the nucleic acids had been extracted was determined by the conventional micro-Kjeldahl technique (Markham, 1942) with a mercuric oxide catalyst (Miller & Houghton, 1945). Protein contents are expressed as mg. Kjeldahl nitrogen/3 mg. dry weight yeast.

Analysis of ribonucleic acids. For the electrophoretic separation of ribomononucleotides, the RNA extract from 40 mg. dry weight of yeast was adjusted to pH 4·0 by careful addition of 10·0 N-KOH, and potassium perchlorate removed by centrifugation (Davidson & Smellie, 1952). Portions (4·0 mg.) of a commercial preparation of yeast RNA (L. Light and Co. Ltd. Colnbrook, Buckinghamshire), which was used as a control, were dissolved in 1·0 ml. of 0·3 N-KOH, incubated at 37° for 18 hr., and the supernatant fluid removed by centrifugation after neutralization with 9·2 N-perchloric acid. The supernatant liquid was then adjusted to pH 4·0 with 10·0 N-KOH, and potassium perchlorate removed by centrifugation. Samples of the solutions (300–500 μl.) were applied as a short band (1·5–2·0 cm.) about 15 cm. from the end of a strip of Whatman no. 3 MM paper (57 cm. × 10 cm.). The paper was soaked in 0·02 M-citrate buffer (pH 3·5), and a potential gradient of 21 V./cm. applied for 7 hr. After removal, the paper was dried with a hair dryer, examined under u.v. radiation (Hanovia 'Chromatolite'), and the positions of the nucleotide spots marked with a pencil. The areas of paper containing the spots were cut out, and the nucleotides eluted from the shredded paper by soaking in 0·01 N-HCl overnight at 37° in a stoppered test tube. The eluate was centrifuged to remove cellulose fibres. The optical density of the eluate was then measured at the appropriate wavelength (adenylic acid, 260 mμ; guanylic acid, 260 mμ (Volkin & Carter, 1951); cytidylic acid, 278 mμ; uridylic acid, 262 mμ (Ploeser & Loring, 1949)), and the molar concentrations of the nucleotides in the eluates calculated from the optical density measurements by using the appropriate millimolar extinction coefficients. Controls of adenylic acid, guanylic acid, cytidylic acid and uridylic acid were run with each electrophoretogram.

Ribonucleobases were obtained by perchloric acid oxidation of the nucleotides. The extract of RNA mononucleotides from 40 mg. dry weight yeast was acidified to pH 5 and evaporated to dryness on a boiling water bath. This was followed by the addition of 0·2 ml. of 12·0 N perchloric acid, after which the solution was heated on the boiling water bath for 70 min. On cooling, 0·2 ml. distilled water was added, and the perchloric acid neutralized by addition of 10·0 N-KOH. The supernatant liquid was then acidified with pure HCl to 2·0 N-HCl, heated in a boiling water bath for a further 5 min. and cooled, after which the precipitate of potassium perchlorate and carbon was removed by centrifugation. This precipitate was washed with 0·1 N-HCl and the washings combined with the original extract. This was then applied as a band (1·0–1·5 cm.) on Whatman no. 3 MM paper, and examined by descending chromatography, using a solvent of *iso*propanol + conc. HCl + water (68 + 16·4 + 15·6; Wyatt, 1951). The paper was irrigated for 40 hr. at room temperature, dried, and examined beneath u.v. radiation. The nucleobases appeared as discrete absorbing spots, the guanine spot being easily distinguished by its bluish tinge. The appropriate areas of paper were cut out, and the nucleobases eluted from the shredded paper by soaking overnight at 37° in 0·10 N-HCl. The optical densities of the eluates were measured at the appropriate wavelengths (adenine, 260 mμ; guanine, 250 mμ; cytosine, 275 mμ; uracil, 260 mμ; Wyatt, 1951), and the molar concentrations of nucleobases calculated by using the appropriate millimolar extinction coefficients. Controls of adenine, guanine, cytosine and uracil were run with each chromatogram.

RESULTS

Effect of biotin deficiency on the concentrations of nucleic acids, protein and acid-soluble u.v.-absorbing substances in yeast

Cultures of the yeast, grown in media containing either an optimal ($8{\cdot}0 \times 10^{-10}$M) or a suboptimal ($0{\cdot}4 \times 10^{-10}$M) concentration of biotin, were removed at intervals and, after growth had been measured, the yeast was washed and analysed for DNA, RNA, protein and acid-soluble u.v.-absorbing substances. The results are shown in Figs. 1 and 2. Under conditions of biotin deficiency, growth of the yeast was restricted and, after *c.* 120 hr. of incubation, the biotin-deficient yeast was coloured pink instead of the usual creamy-white (Chamberlain *et al.* 1952). This restriction in growth and change in colour of the yeast was accompanied by marked changes in the concentrations of DNA, RNA, protein and acid-soluble u.v.-absorbing substances. After an initial slight decrease, the concentrations of DNA in both types of yeast remained constant throughout the period of growth, although under conditions of biotin deficiency, the concentration was significantly lower than in the yeast (biotin-optimal) grown in medium containing an optimal concentration of biotin. The sequence of changes observed in the RNA content of the biotin-optimal yeast was similar to that previously reported by other workers (Di Carlo & Schultz, 1948). In the biotin-deficient yeast, however, the concentration of RNA was, by comparison, low and remained so during the observed period of growth. The

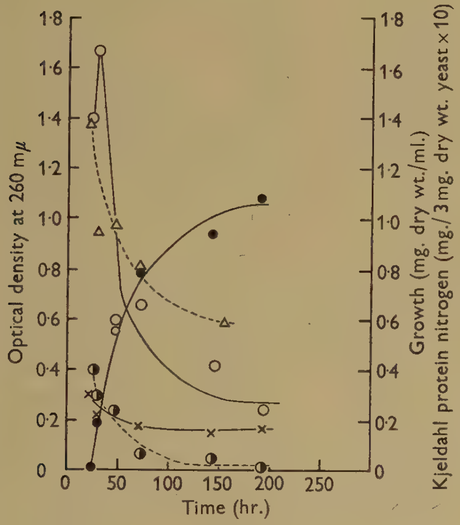

Fig. 1

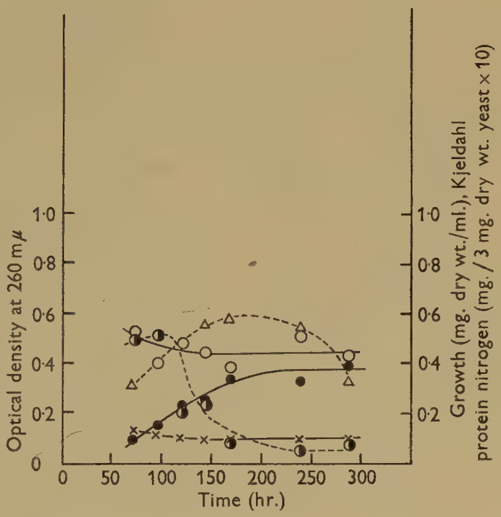

Fig. 2

Figs. 1, 2. Effect of incubation time on growth (●—●, mg. dry wt./ml.) and on the concentrations of DNA (×—×), RNA (○—○), acid-soluble u.v.-absorbing substances (◑—◑) and Kjeldahl protein nitrogen (△—△, mg./3 mg. dry wt. yeast × 10) in yeast grown in media containing either an optimal ($8{\cdot}0 \times 10^{-10}$M) (Fig. 1) or a suboptimal ($0{\cdot}4 \times 10^{-10}$M) (Fig. 2) concentration of biotin. Analyses were conducted on triplicate 3·0 mg. portions of yeast. Concentrations of DNA, RNA and acid-soluble u.v.-absorbing substances are expressed as the optical densities at 260 mμ of extracts from the yeast made up to 3·0, 10·0 and 5·0 ml. respectively with M/15 KH_2PO_4.

concentration of Kjeldahl protein-nitrogen in the biotin-optimal yeast was highest during the very early stages of the exponential phase of growth, but declined steadily as the culture aged. In the biotin-deficient yeast, the protein nitrogen content increased up to 160 hr., when it was approximately half of that in exponential phase biotin-optimal yeast, but, thereafter, gradually declined. The biotin-optimal yeast contained an appreciable amount of acid-soluble u.v.-absorbing substances during the early stages of the exponential phase of growth but on further incubation the concentration declined rapidly and, at the end of the exponential phase, had become extremely small. The biotin-deficient yeast contained significantly higher concentrations of these substances during the early stages of growth although, after *c.* 200 hr., this concentration too had decreased to a low value.

Concentration of acid-soluble u.v.-absorbing substances and stability of RNA in biotin-deficient yeast

The comparatively high concentration of acid-soluble u.v.-absorbing substances in the biotin-deficient yeast during the early stages of growth was obviously of interest in relation to the inability of the yeast to synthesize normal amounts of RNA under conditions of biotin deficiency. It was possible that these u.v.-absorbing substances arose as a result of the breakdown of RNA in the biotin-deficient yeast during extraction with perchloric acid; alternatively, they may have represented purine- and pyrimidine-containing substances that had failed to be polymerized into DNA and RNA. A study was therefore made of the effect of using various acid solutions, including 0·2 N and 1·0 N-perchloric acid, 5 % (w/v) and 10 % (w/v) trichloroacetic acid, and 5 % (v/v) *n*-butanol in M/15 KH_2PO_4 (pH 4·5), to extract these u.v.-absorbing substances from 120 hr. biotin-deficient yeast and from exponential phase (40 hr.) and stationary phase (120 hr.) biotin-optimal yeast. Triplicate 3 mg. portions of washed yeast were taken, and were extracted five or, when necessary, more times with 4·0 ml. portions of the extracting solution at 3° for 5 min. After centrifugation, the supernatant liquid was decanted, adjusted to pH 4·5, and made up to 5·0 ml. with M/15 KH_2PO_4. The optical densities of these extracts were then measured at 260 mμ, with a blank of the appropriate reagent. Removal of the u.v.-absorbing substances was usually complete in five extractions, although complete removal from biotin-deficient yeast with the 5 % aqueous butanol required seven separate extractions. After all the u.v.-absorbing substances had been extracted, the residue was defatted and the RNA estimated in the usual way.

Perchloric acid (0·2 N) extracted all the u.v.-absorbing substances fairly rapidly from biotin-deficient yeast and from biotin-optimal yeast. Consistently larger amounts of u.v.-absorbing substances were extracted from biotin-deficient yeast than from either exponentially growing or stationary phase yeast grown in presence of optimal biotin (Fig. 3). Higher concentrations of perchloric acid (e.g. N) are known to hydrolyse RNA, and this is used as a means of extracting RNA from tissues (Ogur & Rosen, 1950). However, it would seem from the data shown in Fig. 4 that the RNA in biotin-deficient yeast was hydrolysed more quickly by N-perchloric acid as compared with RNA in biotin-optimal yeast.

Many workers have recommended the use of trichloroacetic acid for the extraction of u.v.-absorbing substances before estimation of nucleic acids in tissues (Davidson, Frazer & Hutchinson, 1951; Schmidt & Thannhauser, 1945; Schneider, 1945). Trichloroacetic acid at concentrations of 5 % (w/v) or 10 % (w/v) rapidly extracted the bulk of the acid-soluble u.v.-absorbing substances from the biotin-deficient yeast and from the biotin-optimal yeast. The amounts extracted from biotin-deficient yeast again exceeded those from non-deficient yeast (Fig. 5). Little difference was observed between the amounts extracted by 5 % and 10 % trichloroacetic acid, although a slightly decreased content of RNA in biotin-deficient yeast that had been extracted with 10 % trichloroacetic acid suggested that some of the nucleic acid may have been hydrolysed during extraction.

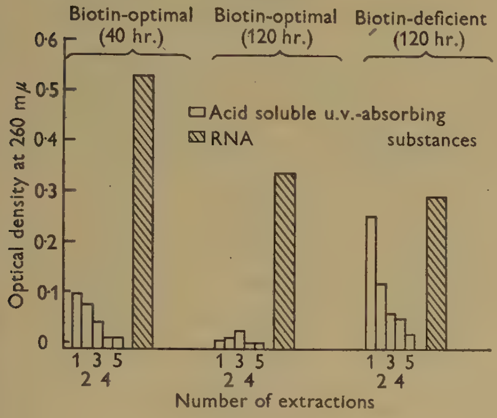

Fig. 3

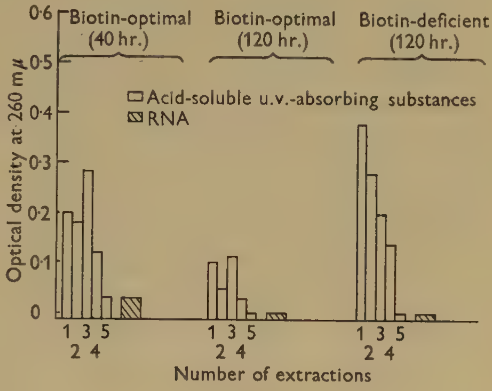

Fig. 4

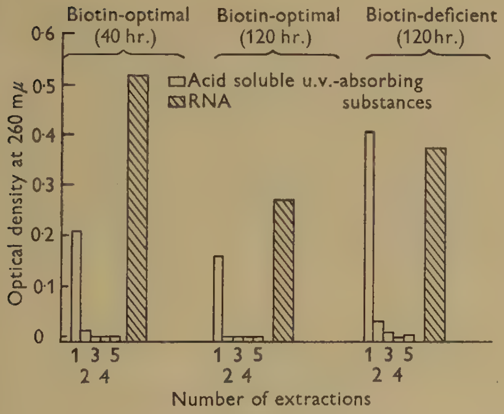

Fig. 5

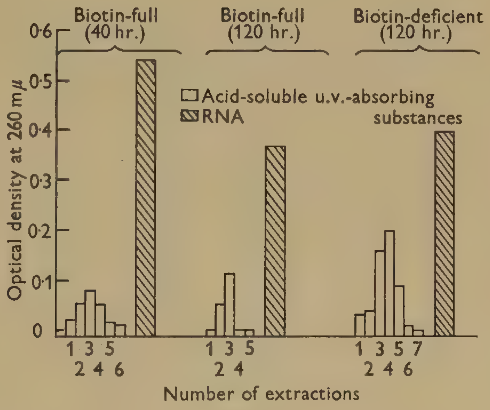

Fig. 6

Figs. 3–6. Extraction of acid-soluble u.v.-absorbing substances from 3·0 mg. portions of biotin-optimal (40 and 120 hr.) and biotin-deficient (120 hr.) yeast with separate 4·0 ml. portions of 0·2N-$HClO_4$ (Fig. 3), 1·0N$HClO_4$ (Fig. 4), 5 % (w/v) trichloroacetic acid (Fig. 5) or 5 % (v/v) *n*-butanol in M/15KH_2PO_4 (Fig. 6). Extracts were adjusted to pH 4·5, made up to 5·0 ml. with M/15 KH_2PO_4, and the optical densities measured at 260 mμ. RNA was extracted as described under Methods.

Extraction of u.v.-absorbing substances by aqueous *n*-butanol was used by Mitchell & Moyle (1951) in their studies on the chemical anatomy of *Staphylococcus* *aureus* (*Micrococcus pyogenes*). This reagent is far milder than either perchloric acid or trichloroacetic acid, and was used in an attempt to minimize possible hydrolytic breakdown of RNA during extraction. Concentrations of *n*-butanol up to 4 % (v/v) in M/15 KH_2PO_4 failed to extract detectable amounts of u.v.-absorbing substances from the yeast. But by using a concentration of 5 % (v/v) butanol in M/15 KH_2PO_4, the u.v.-absorbing substances were extracted, although at least seven separate extractions were required for complete removal of these substances from biotin-deficient yeast (Fig. 6).

Effect of temperature. Further information about the stability of the RNA in biotin-deficient yeast during extraction of the acid-soluble u.v.-absorbing substances was obtained when these extractions were carried out at 21° or 30° instead of at 3°. The results obtained showed that elevation of the temperature had no significant effect on the amounts of u.v.-absorbing substances and of RNA extracted, as compared with the amounts extracted at the lower temperature. Extraction at these elevated temperatures did not affect the amounts of acid-soluble u.v.-absorbing substances extracted from biotin-optimal yeast. Spectrophotometric examination of the various extracts of acid-soluble u.v.-absorbing substances revealed that, in all instances, these showed maximum u.v. absorption at or very close to 260 mμ.

Effect of biotin deficiency on the nucleotide and nucleobase compositions of the yeast RNA

The nucleotide and nucleobase compositions were determined on the RNA extracted from 40 mg. dry wt. yeast. The yeast was extracted with 10 ml. portions of 5 % (w/v) trichloroacetic acid at 3° until the acid-soluble u.v.-absorbing substances had been completely removed; this required five to eight separate extractions. The tissue was defatted by extracting twice with 10 ml. portions of a boiling mixture of 95 % (v/v) ethanol in water (3 vol.) + ether (1 vol.), and the residue treated with 2·0 ml. 0·3 N-KOH for 18 hr. at 37° (Davidson & Smellie, 1952) to hydrolyse polyribonucleotides to soluble mononucleotides. Shorter periods of incubation were tried and, although these were sufficient to allow for the hydrolysis of RNA to acid-soluble nucleotides as detected spectrophotometrically, nevertheless it was shown electrophoretically that incubation for 18 hr. at 37° was necessary

Table 1. *Molar concentrations of adenylic, guanylic, cytidylic and uridylic acids* (*based on adenylic acid* = 10) *in a commercial yeast RNA and in extracts of RNA from* Saccharomyces cerevisiae *grown in media containing an optimal* ($8 \cdot 0 \times 10^{-10}$ M) *or a suboptimal* ($0 \cdot 4 \times 10^{-10}$ M) *concentration of biotin*

		Molar concentration				
Source	Age of culture (hr.)	Adenylic acid	Guanylic acid	Cytidylic acid	Uridylic acid	Ratio: purine/pyrimidine
Commercial yeast RNA	—	10·0	12·64	7·73	12·20	1·13
Biotin-optimal yeast	40	10·0	11·12	8·00	11·31	1·11
	96	10·0	10·6	8·20	11·60	1·03
Biotin-deficient yeast	120	10·0	11·90	7·40	9·76	1·28
	168	10·0	12·70	8·19	12·08	1·12

to obtain complete hydrolysis to the mononucleotides. The nucleotide and nucleobase compositions of the RNA extract were then determined as described under Methods.

The data in Table 1 show the molar concentrations (with adenylic acid expressed as 10) of RNA nucleotides in a sample of commercial yeast RNA and in the RNA from *Saccharomyces cerevisiae* grown in media containing either an optimal or a suboptimal concentration of biotin. These results show the molar ratio of purine to pyrimidine nucleotides ranged from 1·00 to 1·15 in the commercial yeast RNA and in biotin-optimal yeast during the exponential and stationary phases of growth. In biotin-deficient yeast from 120 hr. cultures, the ratio was slightly but consistently higher, the average value obtained being *c.* 1·28; but in yeast from 7-day biotin-deficient cultures, the ratio had decreased to within the range 1·00–1·15. Closely similar results were obtained when the ratio of purine to pyrimidine bases in the yeast RNA was determined. As shown in Table 2, this ratio was in the range 1·00–1·10 in all of the samples of RNA studied, with the exception of that obtained from 120 hr. cultures of biotin-deficient yeast in which it averaged 1·23.

Table 2. *Molar concentrations of adenine, guanine, cytosine and uracil (based on adenine* = 10) *in a commercial yeast RNA and in extracts of RNA from* Saccharomyces cerevisiae *grown in media containing an optimal* ($8{\cdot}0\times 10^{-10}$ M) *or a suboptimal* ($0{\cdot}4\times 10^{-10}$ M) *concentration of biotin*

Source	Age of culture (hr.)	Molar concentration				Ratio: purine/pyrimidine
		Adenine	Guanine	Cytosine	Uracil	
Commercial yeast RNA	—	10·0	10·10	8·07	11·03	1·05
Biotin-optimal yeast	40	10·0	11·36	7·91	11·50	1·10
	96	10·0	11·14	8·50	11·40	1·04
Biotin-deficient yeast	120	10·0	11·60	7·56	10·10	1·23
	168	10·0	11·60	9·10	11·90	1·02

DISCUSSION

Since purine- and pyrimidine-containing nucleotides (e.g. ATP, DPN, coenzyme A) and polynucleotides (nucleic acids) are essential components of all living cells, it is to be expected that any metabolic stress which causes a derangement in the biosynthetic processes leading to the formation of purines or pyrimidines will result in the decreased synthesis of nucleotides and nucleic acids. Although the presence of diminished amounts of total purine in biotin-deficient micro-organisms has not been demonstrated directly, several workers have obtained evidence that biotin is concerned in the synthesis of these nitrogenous bases. The effects of this biotin-conditioned purine deficiency on the synthesis of certain nucleotides in biotin-requiring micro-organisms has already been reported. Thus, Katsuki (1959*a*, *b*) has shown that biotin-deficient *Piricularia oryzae* and *Bacillus macerans* contained diminished amounts of ATP, DNP and coenzyme A, and Rose (1960*b*) reported that the excretion of nicotinic acid and nicotinic acid adenine dinucleotide, two biosynthetic precursors of pyridine nucleotides which appear in the culture medium during growth of *Saccharomyces cerevisiae* under conditions of biotin deficiency, is suppressed on adding adenine to the medium. The results obtained in the present investigation showed that biotin deficiency during growth of a strain of *S. cerevisiae* had a profound effect also on the synthesis of nucleic acids. It was somewhat surprising to discover that, during the early stages of growth, RNA in the biotin-deficient yeast contained an abnormally high content of purine, in view of the adverse effect of biotin deficiency on purine biosynthesis. There was further evidence of a difference between the RNA from 5-day biotin-deficient cultures and that from other cultures, in that the former was more readily hydrolysed by N-perchloric acid. This may indicate a certain instability in structure, a reflexion perhaps of the slightly abnormal base ratio. It is important to note, however, that the RNA extracted from the yeast was heterogeneous and consisted of a mixture of ribosomal, soluble and nuclear RNA. The slight difference in the overall base ratio of the mixture might then be caused by a more significant variation in the base ratio of one of these RNA fractions.

This diminution in the amounts of nucleic acids synthesized under conditions of biotin deficiency was accompanied, during the early stages of growth, by a significant increase in the concentration of intracellular acid-soluble u.v.-absorbing substances which, since they absorbed maximally at approximately 260 mμ, were taken to be purine- and pyrimidine-containing substances. The substances were detected initially in extracts made with the strong acids perchloric and trichloroacetic acids, and it was possible that they represented products from the acid degradation of RNA and DNA. When it was discovered, however, that these u.v.-absorbing substances were also extracted, albeit more slowly, with aqueous *n*-butanol at pH 4·5, a much less drastic reagent, then it was assumed that they were present in the yeast in the soluble state and that they did not represent artefacts of extraction. Some evidence to support this contention came from the discovery that the amounts of u.v.-absorbing substances extracted with acid did not increase significantly when the temperature of extraction was raised from 3° to 21° or 30°, as might have been expected had they arisen as the result of hydrolysis of RNA. No analyses of the composition of this acid-soluble fraction were made in the present study, so it is not known what type of purine- or pyrimidine-containing compounds were present. But at least two -u.v.-absorbing purine precursors, 5-amino-imidazole riboside (Moat *et al.* 1956; Lones, Rainbow & Woodward, 1958) and hypoxanthine (Chamberlain & Rainbow, 1954), are known to be excreted by *Saccharomyces cerevisiae* growing under conditions of biotin deficiency, so that it is likely that these also accumulate in the cells. It is possible too, that ribonucleotides are present in this fraction since these are known to accumulate intracellularly under conditions of decreased RNA synthesis. This has been demonstrated, for example, with a strain of *Escherichia coli* following addition to the culture of the purine analogue 6-azauracil (Skoda & Sorm, 1958).

The role of RNA in protein synthesis is well known, and there is also some reason for believing that protein synthesis is essential for the synthesis of RNA. The results obtained in the present study showed that, under conditions of biotin deficiency, synthesis of total protein by *Saccharomyces cerevisiae* was markedly diminished, and it is possible that this was a result, at least in part, of the decrease in the amount of RNA synthesized and of the formation of possibly abnormal RNA. But for protein synthesis to take place, it is also essential to have in the cell an adequate reservoir or pool of amino acids as well as sufficient energy, in the form of ATP, to activate these amino acids. It is probable, however, that neither of these requirements is met in biotin-deficient yeast, for, not only does biotin-deficient *S. cerevisiae* contain reduced amounts of ATP (Dr M. H. Briggs, personal communication), but it has also been found to contain decreased concentrations of water-soluble ninhydrin-positive substances (Mr A. L. S. Munro, unpublished observations) as compared with biotin-optimal yeast. It would appear, therefore, that there are several metabolic deficiencies contributing towards this overall reduction in protein synthesis.

The diminution in the amount of protein synthesized in biotin-deficient yeast must clearly affect the enzymic activities of the yeast. It is possible that the production of only certain enzymes is affected under conditions of biotin deficiency, which would explain why only a limited number of enzymic activities have been reported to be impaired in yeast grown under this metabolic stress. On the other hand, biotin deficiency may have a non-specific effect on protein synthesis, with the result that production of all of the enzymes in the cell is decreased to about the same extent. Then the deficiency might only be observed in those metabolic reactions for which the enzymes are present in rate-limiting concentrations.

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The Cultivation of D-Galactosamine-metabolizing Lactic Acid Bacteria

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SUMMARY

Media have been devised containing tracheal hydrolyzates or D-galactose as an economical means for the cultivation of large quantities of galactosamine-metabolizing bacteria. With these media *Lactobacillus bulgaricus* and *L. casei* showed good growth; dried preparations were able to utilize galactosamine to produce ammonia and lactic acid.

INTRODUCTION

The wide distribution and importance of glucosamine and galactosamine have been amply reviewed (Kent, 1957; Roseman, 1959), and despite the considerable work done to elucidate the metabolism of glucosamine, little has been reported about the metabolism of galactosamine (Roseman, 1959). This communication describes cultural studies resulting in the production of large numbers of galactosamine-metabolizing bacteria. These included the study of bacteria grown in a medium containing bovine tracheal hydrolyzates as a source of D-galactosamine. Ammonia production and lactic acid formation by dried preparations of lactic acid bacteria, as well as the ability to support growth, were used as criteria of galactosamine utilization.

METHODS

Cultures and media. The following organisms were tested for their ability to grow on D-glucosamine or D-galactosamine: *Streptococcus faecalis* R. ATCC 8043, *S. liquifaciens*, *S. salivarius* ATCC 9756, *Lactobacillus bulgaricus* ATCC 521, *L. buchneri*, *L. casei* ATCC 7469, *L. plantarum* ATCC 8014, *Leuconostoc mesenteroides* P-60 (*Pediococcus*; Garvie, 1959), *Pediococcus cerevisiae*. These cultures were maintained in stabs of liver powder + yeast extract + glucose agar.

The inocula were prepared by seeding Micro Inoculum Broth (Difco) from stab cultures. The cultures after growth for *c.* 18 hr. were centrifuged and washed twice with sterile water. In some cases the inocula were carried over several transfers in medium B (see below), containing glucose, glucosamine or galactosamine at 5 mg./ml. medium, before testing.

For growth studies the inocula were diluted with sterile water to an optical density of 0·4 at 660 mμ in a Beckman Model B spectrophotometer with a light path of 18 mm. One drop of this suspension was then used to inoculate tubes containing 2–6 ml. of the test medium as described in the text.

The medium B was prepared as follows: Casamino acids (Difco), 5 g.; DL-tryptophan, 0·03 g.; L-cysteine.HCl, 0·2 g.; K_2HPO_4 and KH_2PO_4, 0·5 g. each; sodium acetate.$3H_2O$, 10 g.; NH_4Cl, 3 g.; $MgSO_4.4H_2O$, 0·01 g.; $FeCl_3$, 0·03 g.; yeast extract, 3 g.; adenine sulphate, guanine HCl, and uracil, 0·01 g. each; distilled water, 1000 ml.; adjusted to pH 6·8.

Freshly prepared neutralized hexosamine solutions were sterilized by filtration through UF sintered glass filters and added aseptically to previously autoclaved medium.

Preparation of bovine trachea hydrolyzates. Calf trachea, obtained frozen from a slaughterhouse, was cleaned by removing superficial tissues and fat and then passed twice through a meat grinder. The ground trachea was stored at −12° until used. Eighty g. of ground trachea were suspended in 100 ml. of 2 or 4N-hydrochloric acid or 2 or 4N-sulphuric acid and heated in free-flowing steam (100°) for either 7 or 17 hr. The conditions of hydrolysis were arbitrarily chosen. The hydrolyzates were cooled to 5° and solid fat filtered off. The hydrochloric acid-digested material was adjusted to pH 6·8 with sodium hydroxide. The sulphuric acid-digested material was treated with 95% of the amount of barium hydroxide calculated to neutralize all the acid present. The precipitated barium sulphate was removed by filtration and the filtrate adjusted to pH 6·8 with sodium hydroxide.

Chemical determinations. Ammonia was determined by the microdiffusion technique of Conway & Byrne (1933) followed by nesslerization. Lactic acid was measured by the procedure of Barker & Summerson (1941).

D-Galactosamine.HCl and D-glucosamine.HCl were obtained from Mann Research Laboratories (136 Liberty Street, New York, New York, U.S.A.). Each yielded one reducing spot before and after ninhydrin treatment according to the method of Stoffyn & Jeanloz (1954).

Preparation of dried organisms. Dried *Lactobacillus bulgaricus or L. casei* were prepared from cultures grown in medium B which had been supplemented with glucose, glucosamine, galactose or galactosamine to 30 μmole/ml. medium. The flasks were inoculated as follows: for glucose or galactose-containing medium, organisms grown in inoculum broth were used; for flasks containing the amino sugars, organisms grown for several transfers in glucosamine or galactosamine were used. After 28 hr. of growth, the organisms were harvested by centrifugation, washed twice and dried by lyophilization. Lyophilized *L. bulgaricus* and *L. casei* were also prepared from cultures grown on trachea hydrolyzed in 4N-H_2SO_4 for 7 hr. and prepared with glucose supplements as described in the text.

RESULTS

The growth of the organisms

All of the organisms tested with glucose as substrate reached maximum growth at 24 hr.; however, the organisms which grew on the hexosamines required almost 35 hr. to achieve maximum growth. Except for *Lactobacillus buchneri* which showed no growth, all of the organisms grew well on glucosamine. With galactosamine only *L. mesenteroides*, *L. bulgaricus* and *L. casei* achieved more than minimal growth. *L. casei* and *L. bulgaricus* grew well on both amino sugars; these two organisms were studied further.

The growth-supporting activities of glucose, glucosamine or galactosamine were then investigated. Growth on each of these substrates was followed turbidimetrically during 36 hr. To decrease the lag-time for *Lactobacillus casei*, it was grown in a galactosamine-containing medium for several transfers before the test. For both lactobacilli, growth with glucose or glucosamine was superior to growth with galactosamine, at least up to 30 μmole substrate/ml.

Since results such as these raised the question of whether growth on galactosamine was due to mutation or adaptation, a series of plating experiments were designed in which organisms from one glucose supplemented tube were plated on each of three groups of ten agar plates supplemented with 30 μM/ml. of glucose, galactose or galactosamine. In the case of *Lactobacillus casei*, analysis of the data by Fisher's 't' test showed no significant differences between any of the substrates, indicating that the processes were adaptive. For *L. bulgaricus*, however, while glucose and galactose showed no significant differences by the 't' test, on plates containing galactosamine the mean counts were approximately one-half those found in the other two cases.

Some authors (Levene, 1916; Meyer & Rapport 1951) have reported that cartilage is rich in galactosamine. This suggested the use of tracheal hydrolyzates as an economical source for culture media containing this amino sugar. Preparations of hydrochloric acid- or sulphuric acid-hydrolyzed bovine trachea were studied to determine their efficacy as a galactosamine source for the growth of large quantities of organisms. Different conditions of hydrolysis were studied; the results obtained with sulphuric acid-hydrolyzed trachea with *Lactobacillus casei* are shown in Table 1. Hydrolyzates were supplemented with 1% (w/v) yeast extract. It is evident that with 2N- and 4N-sulphuric acid, the 7-hr. hydrolyzates were better than the corresponding 17-hr. hydrolyzates. For *L. bulgaricus* the growth response on the four hydrolyzate media was equivalent to that obtained for *L. casei* grown in the medium containing the 17-hr. hydrolyzates. The hydrolyzates from hydrochloric acid-hydrolyzed trachea were considerably inferior to those treated with sulphuric acid. When the different hydrolyzates containing 1% (w/v) yeast extract were supplemented with various substances, they showed no significant increase in growth over unsupplemented media. The following substances singly, or in mixtures, were used: adenine,

Table 1. *Growth of* Lactobacillus casei *in media containing bovine trachea hydrolyzate*

The sulphuric acid-hydrolyzates contained 1% (w/v) yeast extract in a final volume of 6 ml. After 64 hr. incubation, the organisms were centrifuged, washed once, resuspended in water to 6 ml. and the optical density measured. The hydrolyzates contained solids ranging from 0·082 to 0·088 g./ml.

	Strength of sulphuric acid (N) and period of hydrolysis (hr.)			
	2N; 7	4N; 7	2N; 17	4N; 17
Hydrolyzate (ml.)	Optical density			
1	0·39	0·37	0·35	0·33
2	0·64	0·59	0·55	0·50
3	0·77	0·76	0·72	0·62
4	0·94	0·86	0·80	0·73
5	1·00	1·02	0·85	0·83

guanine, uracil, xanthine, various B vitamins, sodium acetate, ammonium chloride, potassium phosphate, magnesium chloride, ferrous sulphate, tryptophan, cysteine, Casitone, Casamino acids. Although glucose supplementation of the medium increased the growth of both lactobacilli significantly, organisms grown in this manner lost much of their ability to produce ammonia and lactate in the presence of glucosamine or galactosamine.

Experiment with dried organisms

The activities of dried *Lactobacillus casei* and *L. bulgaricus* previously grown on glucose, galactose, glucosamine or galactosamine were tested by determination of their ability to produce ammonia and lactic acid from glucosamine or galactosamine. Ammonia production was taken as an indication of deamination of these amino sugars and lactate production as an indication of the metabolism of their carbon skeletons. Table 2 shows the results of such a study. It can be seen that while glucose- and glucosamine-grown *L. bulgaricus* and *L. casei* had little activity toward galactosamine, they were active toward glucosamine. Galactose and galactosamine-grown *L. bulgaricus* and *L. casei* showed activity for both substrates, although *L. casei* metabolized glucosamine much less actively. With dried organisms the addition of adenosinetriphosphate to any of these assay systems did not increase ammonia or lactic acid production. When dried *L. casei* or *L. bulgaricus* grown in trachea hydrolysates were tested with glucosamine or galactosamine results like those shown in Table 3 were obtained. Supplementation of the media with glucose markedly inhibited the ability of dried preparations to metabolize galactosamine.

Table 2. *Ammonia and lactic acid formation from glucosamine and galactosamine by dried organisms grown in medium B*

The reaction mixture contained: 0·05 ml. M-potassium phosphate (pH 7·0); 2 mg. dried organisms; 5 µmole glucosamine or galactosamine in total volume 1 ml. Tubes were incubated at 37° for 90 min. The reaction was stopped by adding 1 ml. 5 % (w/v) trichloroacetic acid.

	Amino sugar substrate			
	Glucosamine		Galactosamine	
	Product (µmole/ml.)			
Organism	Ammonia	Lactic acid	Ammonia	Lactic acid
L. bulgaricus, glucose grown	2·58	3·04	0·10	0·00
L. bulgaricus, galactose grown	2·00	3·28	2·00	3·82
L. bulgarius, glucosamine grown	4·10	8·10	0·20	0·00
L. bulgaricus, galactosamine grown	4·10	7·30	5·49	10·60
L. casei, glucose grown	2·50	2·82	0·25	0·00
L. casei, galactose grown	2·15	3·36	3·85	7·70
L. casei, glucosamine grown	4·98	9·08	0·55	0·00
L. casei, galactosamine grown	1·54	1·10	5·54	9·28

DISCUSSION

Glucosamine metabolism has been extensively studied and several modes for its microbial breakdown have been reported (Roseman, 1959). On the other hand, galactosamine, aside from its phosphorylation (Cardini & Leloir, 1953; Davidson,

Table 3. *Ammonia and lactic acid formation from glucosamine or galactosamine by dried organisms grown in bovine trachea hydrolyzates supplemented with glucose at different concentrations*

Assay conditions as in Table 2.

		Amino sugar substrate			
		Glucosamine		Galactosamine	
		Product (μmole/ml.)			
Organism	Glucose (%, w/v)	Ammonia	Lactic acid	Ammonia	Lactic acid
L. bulgaricus	0·0	3·38	6·72	2·67	4·26
L. bulgaricus	0·2	4·12	7·08	1·09	1·44
L. bulgaricus	1·0	4·12	7·88	0·40	0·00
L. casei	0·0	4·22	8·46	3·05	4·70
L. casei	0·2	2·11	3·90	0·35	0·00
L. casei	1·0	2·22	3·31	0·50	0·15

1960), has been studied mostly in terms of its uridine derivatives and their subsequent conversion to glucosamine (Glaser, 1959). In the present work we were concerned with the cultural conditions for obtaining a bacterial system that would metabolize D-galactosamine. The *Lactobacillus casei* and *L. bulgaricus* strains examined have such a system, which appears to be inducible. Although preparations of dried organisms made in various ways metabolized glucosamine, only galactose or galactosamine grown-organisms attacked galactosamine. Further investigations with cell-free systems are now in progress in order to ascertain the pathway of galactosamine metabolism.

One of the authors, T. Shiota, was a Research Associate, American Dental Association at the National Institutes of Health.

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Reversible Bicarbonate-Induced Enzyme Activity and the Point of no Return during Morphogenesis in Blastocladiella

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SUMMARY

With synchronous single-generation cultures of the non-filamentous water fungus *Blastocladiella emersonii* the activities of isocitric dehydrogenase, α-ketoglutaric dehydrogenase, and cytochrome oxidase were studied during morphogenesis. The total isocitric dehydrogenase activity per plant increased rapidly following germination of the spore and reached its peak at the point of no return in ontogeny; this increase was about seven times greater than the increase in total α-ketoglutaric dehydrogenase activity. During growth from the spore stage to the stage of irreversibility, the specific activity of isocitric dehydrogenase increased 350 % while that of α-ketoglutaric dehydrogenase decreased 50 %. By removing the external bicarbonate before the point of no return was reached, and thus reversing the morphogenetic pathway, the specific activity of isocitric dehydrogenase, which was in the process of increasing, decreased immediately, while the α-ketoglutaric dehydrogenase activity, which had decreased sharply, increased quickly once again. Similarly, the total activity of isocitric dehydrogenase per plant decreased precipitously while, without reversal, it continued to rise. Conversely, reversal of the morphogenetic path caused an immediate rise in the total α-ketoglutaric dehydrogenase activity per plant while, in the absence of reversal, enzyme activity remained on a level plateau. Thus, removal of bicarbonate induced a new morphogenetic path and, simultaneously, a 250 % increase in the activity of one key enzyme and a 35 % decrease in the activity of a second; on the other hand, the total protein and total activity of two other 'control' enzymes (glucose-6-phosphate dehydrogenase and cytochrome oxidase) remained essentially constant. In contrast to the above, removal of bicarbonate immediately after the point of no return in morphogenesis had no significant effect on these same enzymes. The data provide substantial evidence that a reversible bicarbonate-induced enzyme synthesis is involved in morphogenesis; i.e. in the mechanism of the bicarbonate-induced formation of resistant-sporangial plants in Blastocladiella.

INTRODUCTION

Of the two major morphogenetic pathways that can be taken by a spore of *Blastocladiella emersonii*, the one which leads to a two-celled thick-walled brownpitted resistant sporangial (RS) plant is induced by bicarbonate. In the absence of bicarbonate, a thin-walled papillate ordinary-colourless (OC) plant is formed. Up to a certain point of no return in ontogeny, the young developing RS plant can be made to revert to an OC plant by removing the bicarbonate from its immediate environment. Similarly, before a critical stage in ontogeny, a young OC plant can be induced to form an RS plant by providing it with bicarbonate. But beyond this point of no return, these two diverse morphological pathways become fixed and irreversible. Previous studies have led to the belief that bicarbonate exerts its effect by interfering with decarboxylation sites in the tricarboxylic acid cycle and that, while some enzyme activities of the cycle are consequently lost, a triphosphopyridine nucleotide (TPN)-specific isocitric dehydrogenase remains active and mediates CO_2 fixation via reductive carboxylation of α-ketoglutarate. This bicarbonate trigger mechanism has been discussed in recent reviews (Cantino & Turian, 1959; Cantino, 1960, 1961).

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The purpose of the work to be described here was threefold. (i) To look for precise and direct evidence, by using single-generation synchronous-culture techniques that (*a*) the α-ketoglutaric dehydrogenase system (which was expected to interfere with the bicarbonate trigger mechanism in that it would tend to compete with isocitric dehydrogenase by removing α-ketoglutarate from the carboxylating site) was either lost or severely diminished before the point of no return was reached during the bicarbonate-induced development of an RS plant, while (*b*) the isocitric dehydrogenase (essential for the reductive carboxylation of α-ketoglutarate) either increased in activity or, at least, remained fully functional. (ii) To look for direct evidence that reversal of the morphogenetic pathway (by removal of bicarbonate before the point of no return) caused a corresponding reversal in the activity of these enzymes, believed to be directly implicated in the bicarbonate trigger mechanism, but not in other enzymes more distantly removed from the locus of the bicarbonate effect. (iii) To look for direct evidence that, conversely, removal of bicarbonate after the point of no return in development did not induce significant changes in these same enzymic activities.

METHODS

Procedures for maintenance of stock cultures, and for the growth, sampling and harvesting of large-scale synchronous cultures of *Blastocladiella emersonii* were described previously (Lovett & Cantino, 1960*a*, *b*). Cultures were grown with aeration in a water bath at 24° ± 0·02°. Zoospores were harvested and washed for enzyme studies according to McCurdy & Cantino (1960).

With the exception of zoospores, plants were homogenized in a Serval Omnimixer using 1·0 g. wet weight/10 ml. phosphate buffer (0·05 M; pH 6·8) and 10 g. washed glass beads (200 μ diam., Minnesota Mining and Manufacturing Co.). For zoospores we used 0·2 ml. packed organism (equiv. 25·0 mg. dry wt.)/10 ml. buffer + 10 g. beads. Dry weights were obtained for all samples in order to calculate the per plant data (Lovett & Cantino, 1960*b*). Homogenates were centrifuged for 5 min. at 14,500 *g* and the supernatant fluids used as the enzyme source. The entire procedure was carried out between 0 and 5°. Soluble protein in these preparations was determined by the turbidimetric method of Stadtman Novelli & Lipmann (1951), with slight modification.

In earlier studies (Cantino & Hyatt, 1953), the diphosphopyrimidine nucleotide (DPN)-dependent α-ketoglutaric dehydrogenase in Blastocladiella was assayed spectrophotometrically by following the reduction of nucleotide at 340 mμ or reduction of cytochrome *c* (via carrier nucleotide) at 550 mμ with a cyanide block for the terminal oxidase. In the present experiments, we followed the disappearance of α-ketoglutarate directly by chromatographic isolation and analysis of its 2, 4-dinitrophenylhydrazone (modified from Cavallini & Frontali, 1953; Cavallini & Mondovi, 1957). The specific activity of α-ketoglutaric dehydrogenase was defined as follows: μmole α-ketoglutarate disappearing/30 min./mg. protein in a standard assay containing *c.* 2–8 mg. protein, 100 μmole phosphate buffer (pH 6·8), 0·15 μmole coenzyme A, 0·25 μmole DPN, 50 μmole potassium α-ketoglutarate, in a final volume of 3·0 ml., at 30°. The reaction was stopped by acidification in a boiling water bath.

The activities of isocitric dehydrogenase, cytochrome oxidase, and the glucose-6-phosphate/16-phosphogluconic dehydrogenases (the latter two being equally active in OC plants (Cantino & Horenstein, 1959), and here assayed together as a unit, for simplicity and referred to as glucose-6-phosphate dehydrogenase) were determined spectrophotometrically under standardized assay conditions. In each case the specific activity has been expressed as units/mg. protein. For isocitric dehydrogenase and cytochrome oxidase, one unit was defined as an optical density change of 0·001/min. in the standard assay. For glucose-6-phosphate dehydrogenase, one unit was defined as an optical density change of 0·1/min. in the standard assay. All rates were determined as the average optical density change/min. (corrected for endogenous controls) for the first 3 min. following the addition of enzyme.

The assay for isocitric dehydrogenase contained *c.* 0·45 mg. protein, 100 μmole phosphate buffer (pH 6·8), 0·5 μmole TPN, 0·3 μmole KCN, 10 μmole $MgCl_2$, with and without 20 μmole sodium isocitrate, in a final volume of 3·0 ml., and the optical density change followed at 340 mμ. The assay for cytochrome oxidase contained *c.* 0·7 mg. protein, 100 μmole phosphate buffer (pH 6·8), 0·068 μmole cytochrome *c* (reduced with $Na_2S_2O_3$ followed by aeration to remove SO_2), with and without 0·3 μmole KCN, in a final volume of 3·0 ml., and the optical density change followed at 550 mμ. The glucose-6-phosphate dehydrogenase assay contained *c.* 0·06 mg. protein, 50 μmole phosphate buffer (pH 7·0), 10 μmole $MgCl_2$, 0·5 μmole TPN, with and without 30 μmole glucose-6-phosphate, in a final volume of 3·0 ml., and the optical density change followed at 340 mμ.

The values per plant were calculated from the known dry weights used in the experiments and the dry weight of individual plants at various stages in ontogeny as previously described (Lovett & Cantino, 1960*b*).

RESULTS

The activity of isocitric dehydrogenase, α-ketoglutaric dehydrogenase, and cytochrome oxidase during the growth of an RS plant

The total activity per plant of the isocitric dehydrogenase increased quickly during the growth of an RS plant up to its point of no return and reached a value almost 7000 times that of the spore, while net synthesis of the α-ketoglutaric dehydrogenase activity increased much less rapidly and reached a final value which was only about one-tenth of this amount (Fig. 1). During this same developmental period, the specific activity of the isocitric dehydrogenase increased *c.* 350 % while, on the other hand, the specific activity of α-ketoglutaric dehydrogenase increased slightly and then actually decreased to about 50 % of its original value in the spore (Fig. 2). The activity (not plotted) of the terminal cytochrome oxidase of *Blastocladiella emersonii* followed a course essentially identical with that of α-ketoglutaric dehydrogenase. The final activity of both α-ketoglutaric dehydrogenase and cytochrome oxidase in fully mature RS plants is essentially zero (Cantino & Horenstein, 1955).

Changes in the reversibility of enzymic activity before and after the point of no return during morphogenesis

Plants of *Blastocladiella emersonii* were grown up to a stage (32 hr. at 24°) just preceding the point of no return, removed from the medium, freed from bicarbonate by washing, and then resuspended and agitated in water for 4 hr. at 24°. Under these conditions, such plants reverted to the alternative morphogenetic pathway and, by 36 hr., were ready to form papillae; i.e. they were fully mature thin-walled OC plants. Assays for enzymic activity in 32 hr. RS plants and the 36 hr. OC and RS plants derived therefrom (i.e. reversed and non-reversed, respectively) revealed that the morphological shift to an OC plant was associated with a pronounced and rapid enzymic shift; the specific activity of the α-ketoglutaric dehydrogenase,

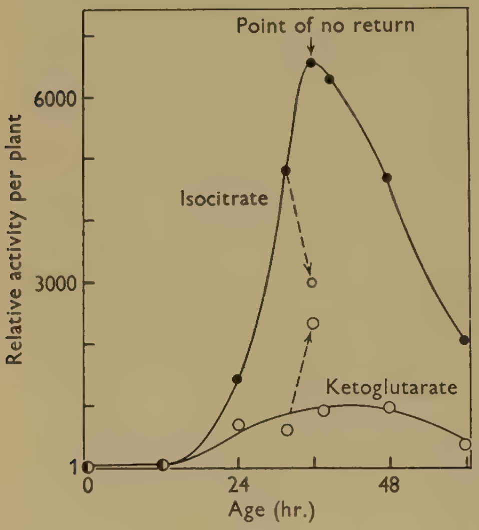

Fig. 1

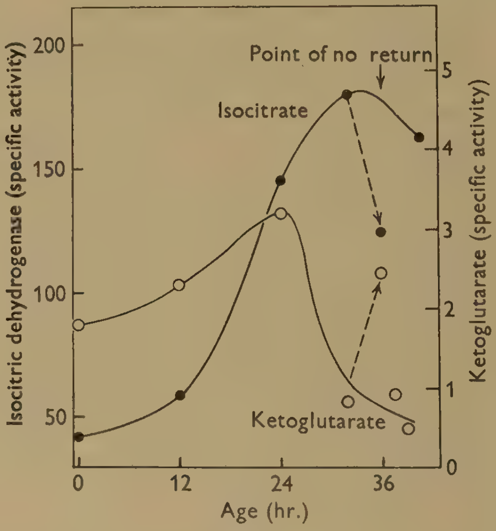

Fig. 2

Fig. 1. The total activities of isocitric dehydrogenase and α-ketoglutaric dehydrogenase per RS plant at different stages of development. The total activities per plant at the spore stage (α-ketoglutaric dehydrogenase, $1{\cdot}934 \times 10^{-7}$ unit; isocitric dehydrogenase, $4{\cdot}52 \times 10^{-6}$ unit) were assigned values of 1·0, and all other total activities at different stages of development were related to the spore level (see Methods for details). The dotted lines represent the changes in activity induced by the removal of bicarbonate.

Fig. 2. The specific activities of isocitric dehydrogenase and α-ketoglutaric dehydrogenase at different stages of development of an RS plant (see Methods for details). The dotted lines represent the changes induced by the removal of bicarbonate.

which reached a low value at 32 hr., rose quickly once again; the specific activity of the isocitric dehydrogenase, which was in the process of ascending rapidly toward its peak, dropped sharply (Fig. 2). Even more pertinent from an interpretative point of view (see Discussion) were the per plant data, which revealed similarly striking reversals in total enzymic activity (Fig. 1). Under these identical conditions, however, the cytochrome oxidase and the glucose-6-phosphate dehydrogenase activities did not change significantly (Table 1). The significance of these observations is emphasized by analyses of RS plants subjected to the same treatment (i.e. removal of bicarbonate) after the point of no return in morphogenesis had been reached. At this later stage in ontogeny none of these enzymic activities changed appreciably following incubation for 4 hr. in the absence of bicarbonate.

Table 1. *Enzymic activities before and after a four-hour incubation of young RS plants of* Blastocladiella emersonii *in water*

	Cytochrome oxidase	Glucose-6-phosphate dehydrogenase
	Specific activity	
32 hr. RS plants before incubation	16·9	6·9
32 hr. RS plants after incubation	16·5	8·1

See Methods for details.

DISCUSSION

The bicarbonate trigger mechanism for morphogenesis, as originally visualized from the results of experiments with multiple-generation cultures of Blastocladiella involves the gradual cessation of a weakly functional tricarboxylic acid cycle and, concomitantly, increased carboxylation of α-ketoglutarate via a TPN-specific isocitric dehydrogenase as the developing plant becomes committed to the formation of a resistant sporangial (RS) plant (Cantino, 1961). More recently, data derived from experiments with synchronous, single-generation cultures have corroborated and amplified these notions (Cantino & Lovett, 1960; Lovett & Cantino, 1960*a*,*b*; McCurdy & Cantino, 1960). In this report we have now provided more precise and direct evidence that the isocitric dehydrogenase activity required for carboxylation does indeed, increase quickly and many fold, while the enzyme system which would compete with it for α-ketoglutarate by oxidatively decarboxylating this substrate (α-ketoglutaric dehydrogenase and an associated terminal cytochrome oxidase) decreases sharply. It is particularly noteworthy that during the development of an RS plant, bicarbonate also induces, simultaneously, an immediate exponential synthesis of isocitritase, a crucial enzyme required for removal of the isocitrate following its formation from α-ketoglutarate via isocitric dehydrogenase. This does not occur in the absence of bicarbonate during the early stages of development in an ordinary colourless (OC) plant (McCurdy & Cantino, 1960; Cantino, 1961).

An appreciable accumulation of evidence (Cantino & Turian, 1959; Cantino, 1960, 1961) suggests that these bicarbonate-induced changes in enzyme activity are intimately related in cause and effect fashion to the differentiation of an RS plant. On the basis of previous arguments (Cantino, 1961) and the data here reported, we are now strongly tempted to conclude that these alterations in measurable enzymic activity actually reflect bicarbonate-induced reversible changes in enzyme synthesis and enzyme degradation.

Before the point of no return in ontogeny, the bicarbonate-triggered morphogenetic mechanism leading to an RS plant remains plastic and reversible; the activities of two of the three key enzymes (α-ketoglutarate dehydrogenase, isocitric dehydrogenase) presumed to be most directly involved in this mechanism, seem to be equally plastic and reversible, responding quickly to the addition or removal of bicarbonate; the third, isocitritase, has not yet been tested for reversibility. Beyond the point of no return, when morphogenesis becomes irreversible and continues to completion, the same two enzyme systems lose their plasticity. These observations seem particularly significant in the light of the obvious lack of pliability in the activities of other enzyme systems, namely the TPN-specific glucose-6-phosphate dehydrogenase and the terminal cytochrome oxidase, neither of which is directly involved in the bicarbonate trigger mechanism, nor is altered by the removal of bicarbonate, either before or after the irreversible point in morphogenesis.

The per plant data, however, provide the greatest support for our argument. While the morphogenetic shift is associated with a several-fold increase in the total α-ketoglutaric dehydrogenase activity per plant and a sharp decrease in the total isocitric dehydrogenase activity per plant, neither the total cytochrome oxidase per plant, the total glucose-6-phosphate dehydrogenase activity per plant, nor the total protein per plant changes significantly. Thus the changes in enzyme activity cannot be artifacts resulting from an alteration of the total protein content of the fungus which would result in an apparent unidirectional change in specific activity. While it is always possible that some kind of ill-defined 'masking effect' has been induced by the removal of bicarbonate, this alternative explanation seems improbable.

There is little if any published evidence to show that induced enzymic synthesis is related in a cause-and-effect fashion to morphogenesis (Markert, 1958). In the light of these and earlier (Cantino, 1961) experiments, it seems very likely that, in *Blastocladiella emersonii*, bicarbonate does induce both synthesis and degradation of adaptive enzymes whose activities, in turn help to initiate and direct morphological differentiation.

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The Endogenous Metabolism of *Euglena gracilis*

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SUMMARY

The endogenous respiratory rate of non-photosynthetic *Euglena gracilis* var. *bacillaris* is remarkably constant under varied conditions of growth and incubation. After 60–90 min. of slightly more rapid respiration, the endogenous oxygen uptake averages about 6·5 μl. O_2/million cells/ hr., and this rate can be maintained for at least 26 hr. The respiratory quotient of the endogenous metabolism is 1·0. Fluoroacetate inhibits endogenous oxygen consumption about 70 %. A variety of evidence suggests that the polysaccharide paramylum is the major endogenous substrate.

Tracer experiments indicate that the endogenous metabolism continues during the oxidation of exogenous substrates, but that the assimilated substrate mixes with, and dilutes the endogenous reserves. The time-course of this dilution process indicates that the most recently assimilated reserves are the first to be oxidized.

INTRODUCTION

In the absence of exogenous substrate, cells of *Euglena gracilis* var. *bacillaris* (non-photosynthetic strain) have a respiratory rate roughly one-quarter that on a substrate such as acetate or ethanol. Previous manometric experiments indicated that this endogenous respiration continues unabated during the metabolism of exogenous acetate (Wilson & Danforth, 1958). The experiments described here were designed to provide further information concerning this endogenous metabolism and its relation to the metabolism of exogenous substrates.

METHODS

Experiments were done on the same non-photosynthetic strain of *Euglena gracilis* var. *bacillaris* used in previous studies (Danforth, 1953; Danforth & Wilson, 1957; Wilson & Danforth, 1958). Cells were grown at *c.* 20° in a medium composed of (w/v) Bacto-Tryptone (Difco), 0·25 %; with Na acetate $3H_2O$, 0·3 % (w/v) at pH 7·3; or ethanol, 0·3 % (v/v) at pH 3·5 as carbon sources. Several experiments on the rate of endogenous oxygen consumption were performed on cells grown at 24–25° in the chemically defined medium of Cramer & Myers (1952), with acetate, 0·5 % (w/v); or succinate, 0·5 % (w/v) at pH 7·0 or fumarate, 0·5 % (w/v); malate, 0·5 % (w/v); or α-ketoglutarate, 0·5 % (w/v) at pH 5·0 as sole carbon sources. Cells were harvested and washed by centrifugation before experimental use. These procedures have been described in more detail in the previous publications cited above.

'Partially-labelled' Euglena was prepared by incubating the cells for 10–20 min. in 0·01 M-phosphate buffer (pH 7·0) containing 5·0–6·4 μmole of Na acetate-2-^{14}C. 'Fully-labelled' cells were prepared by inoculating 1 ml. of Euglena culture into 300 ml. growth medium containing acetate-2-^{14}C. Cells were harvested after 8 days of growth in this medium. The labelled cells were dispersed into experimental media in Warburg flasks or 50 ml. Erlenmeyer flasks equipped with centre wells. Each centre well received 0·5 ml. (Warburg) or 0·1 ml. (Erlenmeyer) of 0·1 M-NaOH. Flasks were stoppered and shaken at 120 cyc./min. Experiments were performed at 21–26°. At approximately hourly intervals, the NaOH was withdrawn from the centre wells and the wells rinsed and refilled with the same volume of fresh NaOH. The entire withdrawal, rinsing, and refilling took less than a minute, so loss of CO_2 during this process was probably less than 2 % of the measured values. Samples of the NaOH withdrawn were spread uniformly on planchets with the addition of ethanol and were dried under an infra-red lamp. Radioactivity was counted with a thin-window Geiger tube for a period of time sufficient to give a total count of 1000 or more. Background was counted to the same degree of accuracy, and was subtracted from all counts. No correction was made for self-absorption, since preliminary experiments had indicated that differences in self-absorption between samples were negligible.

In some experiments samples of labelled Euglena were killed in known volumes of alkaline ethanol (0·01 M-NaOH in 95 % (v/v) ethanol), at the beginning and the end of the experiment. Known volumes of these samples were dried on planchets, and counted to determine the radioactivity of the cells.

Under the experimental conditions described above, there was a certain amount of retention of CO_2 in the incubation medium. To determine whether the incomplete recovery of CO_2 caused any serious error in the results, a number of experiments were performed by a method designed to eliminate the effect of CO_2 retention. In these experiments, samples were removed from the incubation flasks at intervals and centrifuged. The cells were washed and resuspended in fresh medium, and these new suspensions transferred to Warburg flasks whose centre wells contained 0·4 ml. of 0·1 M-NaOH and whose sidearms contained 0·5 ml. of 0·1 M-H_2SO_4. After incubation for the desired period, the sidearms were tipped, killing the Euglena and acidifying the medium. The flasks were shaken for another hour to complete the diffusion of CO_2 to the centre well, and the centre well contents were sampled and counted as before. The experiments shown in Figs. 3 and 6 were done by this method. In every case the results of such experiments were similar to those using the simpler method described above.

Respiration was measured by standard Warburg techniques, at 26°, in a gas phase of air. The respiratory quotient was determined by the acid end-point method described in a previous report (Wilson & Danforth, 1958). Respiration of cells grown in Cramer-Myers medium was measured in the same medium minus carbon source. Tris (2-amino-2′′ hydroxymethylpropane – 1:3-diol) buffer + phosphate (tris + phosphate) or phosphate alone were used as buffers for other respiratory experiments.

To determine the volume of Euglena cells, measured portions of a heavy suspension of Euglena were centrifuged in Hopkins vaccine tubes until the cells were packed in the narrow graduated portions of the tubes. Centrifugation was continued until a pellet of constant volume was achieved. Because the plasticity of the Euglena pellicle permits tight packing, the volume of extracellular water in such pellets was probably small. The number of cells was determined by haemocytometer counts on samples of the same suspension.

Na acetate-2-^{14}C was obtained from the Volk Radiochemical Company, Chicago, Illinois; Na fluoroacetate (Compound 1080, 10% inert material) was the gift of Mr Tull C. Allen, Tull Chemical Company, Inc., Oxford, Alabama, U.S.A.

RESULTS

General characteristics of the endogenous metabolism. As was noted by Von Dach (1942) in the closely related Astasia, Euglena cells often have a slightly higher rate of endogenous metabolism during the first hour or two after harvesting than at later periods. When cells grown in Cramer–Myers medium with acetate, succinate, fumarate, malate, or α-ketoglutarate were incubated at pH 5·0, 5·5 or 7·0, eighteen experiments yielded an average initial endogenous respiration rate of $8{\cdot}2 \pm 1{\cdot}2$ (av. dev.) μl. O_2/million cells/hr. After this initial period of rapid respiration, the rate decreases to a constant level, $6{\cdot}5 \pm 1{\cdot}0$ μl. O_2/million cells/hr. in the same series of experiments. The same change has been noted in cells grown on Tryptone media with acetate or ethanol as growth substrates, and incubated in tris-phosphate buffer. The endogenous respiratory rate was independent of the growth substrate, the presence or absence of a nitrogen source in the incubation medium, the pH of incubation, and the other differences in incubation media described under 'Methods'.

The later, steady rate of endogenous respiration is maintained for long periods of time. In the longest experiment performed, the rate of endogenous metabolism during the 24th to 26th hour was 93% of that during the 3rd and 4th hour. Assuming that the rate of O_2 consumption in this experiment equalled the average rate of 6·5 μl./million cells/hr. (unfortunately the cell count sample in this experiment was lost), the total oxygen consumption during the entire experiment was 7·3 μmole/million cells, equivalent to the complete oxidation of 3·6 μmole of acetate or 1·2 μmole hexose/million cells. Since the respiratory rate was still appreciable at the end of the experiment, it is clear that these are minimum estimates of the endogenous reserves of Euglena cells.

Effect of fluoroacetate on endogenous metabolism. It would be of some interest to determine the pathways and intermediates involved in the endogenous metabolism of Euglena, particularly in view of the metabolic scheme presented in the discussion, which assumes a minimum of interaction between the endogenous metabolism and that of exogenous substrates. Some few data bearing on this problem have been obtained.

One source of such data are experiments involving the inhibitor, fluoroacetate. In higher animals, this substance has been shown to be incorporated, by way of the 'condensing enzyme', into fluorocitrate, which inhibits the enzyme aconitase and thus blocks citrate metabolism (Peters, 1952, 1957). Danforth (1952) found that fluoroacetate inhibited acetate oxidation by Euglena, but was unable to demonstrate the expected accumulation of citrate. Holz (1954), however, was able to show accumulation of large amounts of citrate in fluoroacetate-poisoned Euglena of the same strain. Thus, it seems that the mechanism of fluoroacetate inhibition in Euglena is the same as that in the tissues of higher animals.

Figure 1 shows that 0·001 M-fluoroacetate inhibited endogenous oxygen consumption of Euglena by about 68 %. Further increases in concentration did not result in appreciably greater inhibition. It seems likely, therefore, that the endogenous metabolism occurs, in large part at least, through pathways in which citrate is an intermediate.

The respiratory quotient of the endogenous metabolism. Determination of the respiratory quotient of the endogenous metabolism also yielded some information concerning the nature of the substrates involved, though such data cannot provide positive identification of the substrates. Six experiments, using cells grown on acetate + Tryptone at pH 7·3 and ethanol + Tryptone at pH 3·5, resulted in an average respiratory quotient of 1·05 ± 0·19. The respiratory quotient of the endogenous, like its rate, was independent of the pH value and substrate of growth, and of the composition and pH value of the experimental media.

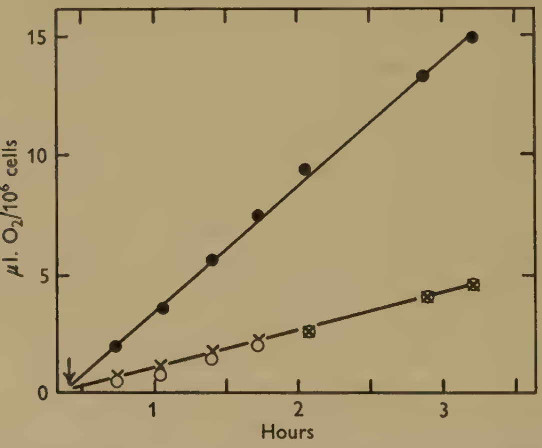

Fig. 1. Effect of fluoroacetate on the endogenous oxygen consumption by Euglena. Warburg flasks contained 17·4 million Euglenas in 3·0 ml. 0·01 M-phosphate buffer (pH 7·0). Temperature was 26°. Time is measured from the time of addition of fluoroacetate; the arrow indicates the time manometer readings were begun. ●, No fluoroacetate; ×, 0·001 M-fluoroacetate: ○, 0·04 M-fluoroacetate.

The respiratory quotient of approximately 1·0 suggests that the endogenous store is carbohydrate in nature.

Interaction of endogenous and exogenous metabolism. Wilson & Danforth (1958) concluded, on the basis of respirometric data, that the endogenous metabolism of Euglena continues unchanged during the oxidation of exogenous acetate. This conclusion was based on indirect evidence; it was assumed that the ratio of acetate oxidized to acetate assimilated in short-term experiments should be independent of the total amount of acetate utilized. It was found that the data fitted this assumption only when corrected for a continuing endogenous metabolism. In the case of ethanol, this criterion did not permit a decision as to whether or not the endogenous metabolism continued.

Blumenthal, Koffler & Heath (1957) showed that the manometric method described above and measurements based on release of radioactive carbon dioxide from ^{14}C-labelled cells may lead to apparently contradictory conclusions concerning the effect of exogenous substrates on endogenous metabolism. It seemed desirable, therefore, to compare results obtained by the tracer method with our earlier respirometric studies. Figures 2 and 3 show the results of two such experiments, in which Euglena which had assimilated small amounts of radioactive acetate were then incubated in the presence and in the absence of exogenous substrate.

In the absence of substrate, the rate of $^{14}CO_2$ production decreases rapidly with time; the decrease follows an approximately logarithmic time-course. This decrease cannot be attributed solely to the decreasing radioactivity of the cells, since at the end of the experiment shown in Fig. 2, the cells retained more than 90 % of their original radioactivity, while the rate of release of the $^{14}CO_2$ had dropped to about 25 % of the initial rate.

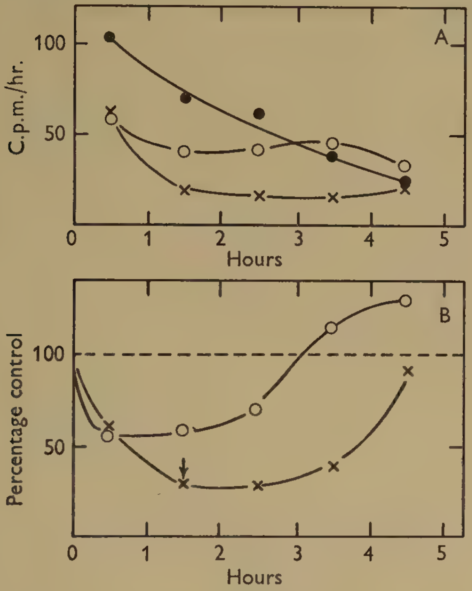

Fig. 2

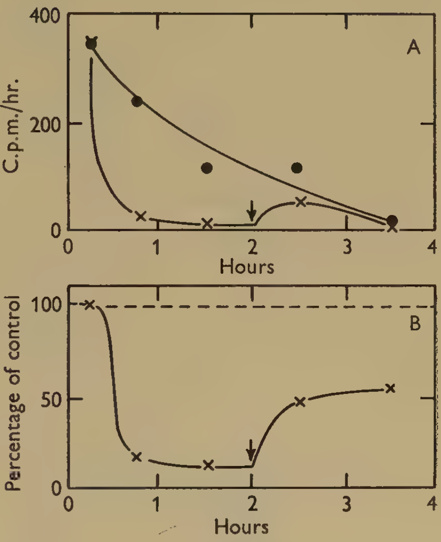

Fig. 3

Fig. 2. Effect of nonradioactive substrate on release of $^{14}CO_2$ by 'partially labelled' Euglena. CO_2 trapped by first method (see 'Methods'). Incubation mixtures contained per flask, 15·3 million Euglenas, 1450 c.p.m., in 1·0 ml. 0·03 M-phosphate buffer (pH 7·0). Temperature was 25–27°. A, Rates of $^{14}CO_2$ release. The average rate of release is plotted in the mid-point of the measuring interval. B, Rates of $^{14}CO_2$ release as % rate in the absence of substrate. ●, No substrate; ×, 20 μmole acetate; ○, 20 μmole ethanol. Arrow indicates the most probable time of acetate exhaustion.

Fig. 3. Effect of non-radioactive acetate on release of $^{14}CO_2$ by 'partially labelled' Euglena. CO_2 trapped by second method. Incubation mixtures contained per flask, 2·0 million Euglenas in 1·5 ml. 0·008 M-phosphate buffer, pH 7·0. Temperature was 26°. A and B, Data plotted as in Figs. 2A and 2B, respectively. Arrow indicates time of removal from acetate. ●, No substrate; ×, 20 μmole acetate/ml.

It is apparent that the presence of exogenous acetate or ethanol markedly reduces the rate of $^{14}CO_2$ production by labelled cells. The course of this 'inhibition' is clearest when the data are plotted as in Figs. 2B and 3B, expressing the rates of $^{14}CO_2$ production in the presence of substrate as percentage of the endogenous rates over the same time intervals. The inhibition develops gradually after addition of the substrate; in the experiment shown in Fig. 3, there was no inhibition during the first half hour after addition of acetate. With time, the rate of $^{14}CO_2$ release recovers toward or beyond the endogenous rate. It seemed probable that the recovery began at the time when the exogenous substrate was exhausted. In the experiment shown in Fig. 2, the probable time of exhaustion of acetate was estimated from the amount of acetate added and the average rate of acetate utilization by Euglena cells in other experiments performed under the same conditions. The vertical arrow in the figure indicates the most probable time of acetate exhaustion. In the experiment shown in Fig. 3, the known time of transfer from acetate-containing to endogenous medium is shown by a similar arrow.

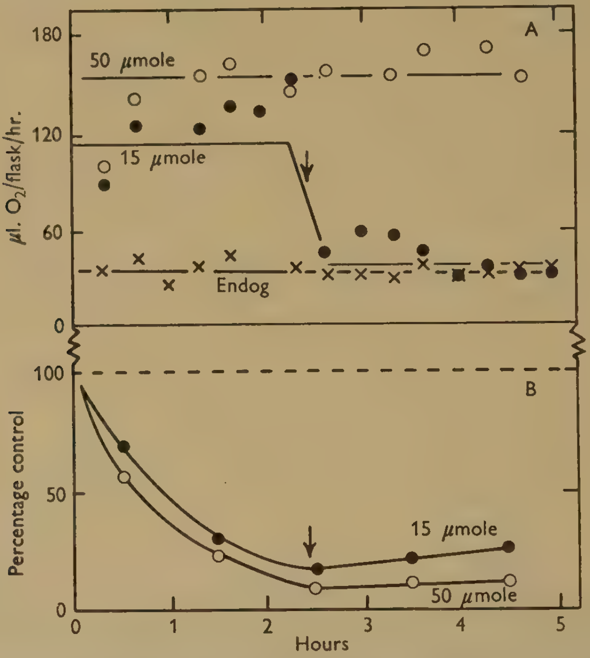

Fig. 4. Effect of nonradioactive acetate on respiration and rate of release of $^{14}CO_2$ by 'partially labelled' Euglenas. CO_2 trapped by first method. Mixtures contained per flask, 6·0 million Euglenas, 4750 c.p.m., in 1·0 ml. 0·008 M-phosphate buffer, pH 7·0. Temperature was 24°. A, Rates of oxygen consumption. B, Rates of $^{14}CO_2$ release as % of rate with no substrate. Figures beside the curves indicate the amounts of acetate added.

It is clear that the decreased rate of $^{14}CO_2$ production persists for as long as several hours after depletion of exogenous substrate. This fact is apparent in Fig. 4, which records an experiment in which rates of oxygen consumption and $^{14}CO_2$ production were measured simultaneously in duplicate flasks. Since the respiratory quotient of both endogenous and acetate (Wilson & Danforth, 1958) is 1·0, the total (labelled plus unlabelled) CO_2 production may be taken as equal to the oxygen consumption. Two initial values of acetate, 15 and 50 µmole, were used. The former was exhausted during the course of the experiment, while the latter was not. As before, the inhibition of radioactive CO_2 production developed gradually and persisted for several hours after the sharp drop in respiratory rate to the endogenous value, which indicates exhaustion of the acetate.

Blumenthal *et al.* (1957) also showed that the apparent effect of exogenous substrates on endogenous metabolism might vary, depending on whether the cells were labelled by brief exposure to radioactive substrate or were grown from a small inoculum entirely on uniformly labelled substrate. In the first case, various endogenous substrates may be labelled to different degrees, while in the second all cellular constituents are uniformly radioactive. For this reason, experiments were performed on Euglena grown through several-hundredfold multiplication on radioactive acetate. Such cells were not strictly 'uniformly-labelled', since the acetate used for growth was labelled only in the methyl carbon, and since the nonradioactive Bacto-Tryptone in the medium probably supplied some carbon to the cells. But such cells certainly more closely approach uniformity of labelling than do those used in the preceding experiments.

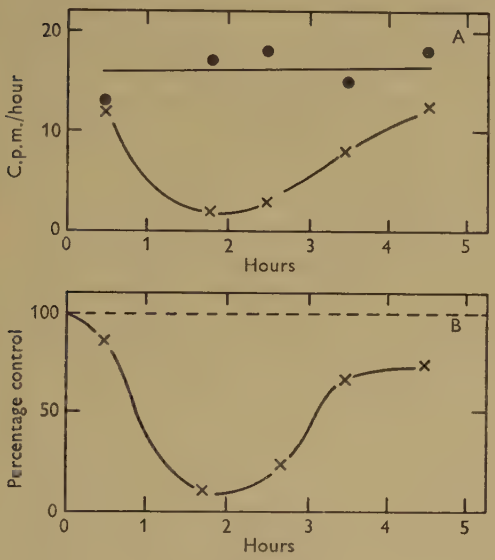

Fig. 5

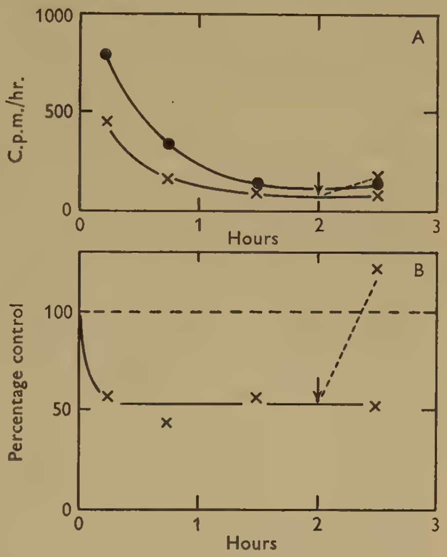

Fig. 6

Fig. 5. Effect of non-radioactive acetate on release of $^{14}CO_2$ by 'fully labelled' Euglena. CO_2 trapped by first method. Incubation mixtures contained per flask, 6·8 million Euglenas, 4380 c.p.m., in 1·0 ml. 0·008 M-phosphate buffer, pH 7·0. Temperature was 21°. A and B, Data plotted as in Fig. 2A and 2B respectively. ●, No substrate; ×, 10 μmole acetate.

Fig. 6. Effect of non-radioactive acetate on release of $^{14}CO_2$ by 'partially labelled' Et-grown Euglena. CO_2 trapped by second method. Incubation mixtures contained per flask, 18·5 million Euglenas in 1·5 ml. 0·008 M-phosphate buffer (pH 7·0). Temperature was 26°. At the time indicated by the arrow, one sample of cells was transferred from acetate medium to endogenous medium (dotted curve). A and B, Data plotted as in Fig. 2A and 2B, respectively. ●, No substrate; ×, 20 μmole acetate/ml.

Figure 5 shows the results of an experiment with such 'fully-labelled' cells. A comparison with Figs. 2 and 5 shows a number of interesting differences. Although the initial radioactivity of the 'fully-labelled' cells was about twice that of the partially labelled, the rate of release of $^{14}CO_2$ was much lower in the fully labelled. This would be expected if newly assimilated acetate is converted mainly into the cellular constituents most readily available as endogenous substrates. This explanation is supported by the fact that the rate of production of radioactive CO_2 by partially labelled cells decreases rapidly with time, while that of the fully labelled cells remains constant over the same period of time.

The effect of exogenous acetate on the release of radioactive CO_2 is, however, the same in fully-labelled cells as in partially labelled. There is the same gradual decrease in rate to about 10–30 % of the endogenous rate, and the same slow recovery following disappearance of the exogenous acetate.

All of the preceding experiments were done on Euglena which had been grown on acetate at pH 7·3, the 'Ac-grown' cells described in previous publications (Danforth & Wilson, 1957; Wilson & Danforth, 1958). Cells grown on ethanol at pH 3·5 ('Et-grown') have much lower rates of acetate metabolism. We were interested, therefore, in comparing the effect of acetate on endogenous metabolism in Et-grown cells with that in Ac-grown cells. Figure 6 shows the results of an experiment similar to that of Fig. 3, but performed with Et-grown Euglena. Two differences are apparent; with Et-grown cells the inhibition develops more rapidly, but the final level of inhibition is less than with Ac-grown cells.

The volume of Euglena cells. The volume of Euglena cells was estimated from a series of twenty-three determinations on four different cell suspensions. The averages for the four suspensions ranged from 1·15 to 1·80 μl./million cells, with a grand average of 1·58 μl. ± 0·12 μl./million cells. This volume is about half that found by Neff (1960) for a green strain of *Euglena gracilis* var. *bacillaris*. Strain differences or differences in culture methods may account for this discrepancy.

DISCUSSION

Effect of substrates on the endogenous metabolism. The utilization of exogenous acetate or ethanol greatly depresses the rate of release of radioactive CO_2 by Euglena previously labelled with radioactive carbon. At first glance, these results seem to indicate that, contrary to our earlier conclusions, the presence of exogenous substrate depresses the rate of endogenous CO_2 production. Two facts, however, suggest that the situation is not quite so simple. First, the rate of release of radioactive CO_2 remains depressed for more than an hour after the substrate has been exhausted. But numerous manometric experiments have shown that, after acetate exhaustion, the respiratory rate returns promptly to the endogenous level, as illustrated in Fig. 4. Secondly, it would be difficult to explain the gradual onset of the inhibition of $^{14}CO_2$ production by any mechanism involving a direct competition between the endogenous and acetate metabolism, since acetate metabolism begins at maximum rate almost immediately upon the addition of acetate. On the other hand both the gradual onset of the inhibition and the persistence of the inhibition after acetate exhaustion can be explained if it is assumed that the rate of total CO_2 production from endogenous sources remains unchanged in the presence of acetate, but that the specific radioactivity of the endogenous reserves is lowered by the assimilation of non-radioactive substrate, thus decreasing the radioactivity of the CO_2 produced from this source.

A metabolic scheme in which such dilution might occur is shown in Fig. 7. In this model, it is assumed that the endogenous reserves are composed of two (or more) components, a 'labile reserve' which is the immediate source of substrate for endogenous metabolism, and a larger pool of 'stable reserve(s)' from which the labile reserve is replenished. It is further assumed that endogenous acetate or ethanol is assimilated directly into the labile reserve, and enters the stable reserve only indirectly. Such a model explains the fact, noted previously, that the most recently-assimilated reserves are most readily available for endogenous metabolism. The decrease with time of the rate of endogenous $^{14}CO_2$ production by partially labelled cells would occur not only because of rapid re-oxidation of the new, radioactive reserves, but also because of the exchange of material between highly radioactive labile reserve and the less active stable reserve.

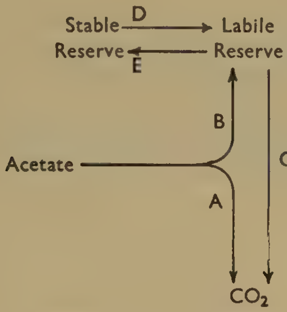

Fig. 7. Hypothetical model of acetate and endogenous metabolism in Euglena.

In this model, then, reaction C is the continuing endogenous metabolism of Wilson & Danforth (1958), reaction A the oxidation of exogenous acetate, and reaction B the assimilation of acetate. When unlabelled acetate is present, the assimilation of non-radioactive carbon directly into the labile reserve would dilute these reserves, lowering the specific activity of the CO_2 produced via reaction C, even though the total rate of CO_2 production from this pathway remained unchanged. The extent of dilution would increase with time, and the dilution would persist for a time after the exhaustion of substrate, in agreement with the experimental findings.

This model also accounts for the fact that 'inhibition' of $^{14}CO_2$ production by acetate is less in Et-grown than in Ac-grown cells, since both reaction A and reaction B (oxidation and assimilation of acetate) are slower in Et-grown cells. The labile reserves would therefore be less diluted by acetate in the case of Et-grown cells. The precise mechanism of the more rapid onset of inhibition in Et-grown cells is not known, but the phenomenon is not inconsistent with the model presented here.

The only essential feature of the model presented is the assumption that the newly-assimilated material 'pools' more rapidly with the immediate substrate of endogenous metabolism than with the remainder of the endogenous reserves. In other respects, the model is probably oversimplified. In particular, it seems doubtful that only two compartments are involved, and that the distinction between labile and stable reserves is a sharp one. A series of many compartments with varying degrees of 'lability' would probably be closer to reality.

Nature of the endogenous reserves. While the present experiments do not permit conclusive identification of the nature of the endogenous reserves, they provide a number of important clues. For example, from the rate and duration of endogenous respiration, it was calculated that the reserves contain at least the equivalent of about 1 μmole of hexose per million cells. From the measured volume of the cells, this would amount to the equivalent of an approximately 0·6 M-hexose solution, a concentration larger than any reasonable estimate of the total osmolar concentration of Euglena cytoplasm. Thus, the bulk of the reserves must be in the form of high molecular weight compounds, or of insoluble materials.

The respiratory quotient of 1·0 suggests that the endogenous reserves are carbohydrate in nature. Paramylum, a polymer of D-glucose, is a likely candidate for the stable reserve substance. It is present in Euglena as granules which are frequently so numerous as to be the most conspicuous cellular inclusions. These granules have been observed to decrease in size and number during prolonged starvation (Gojdics, 1953; Padilla & Buetow, 1959).

Pathway of endogenous metabolism. The effectiveness of fluoroacetate as an inhibitor of endogenous respiration suggests that the endogenous metabolism occurs via the Krebs tricarboxylic acid cycle. Since acetate is also probably oxidized through the Krebs cycle (Danforth, 1953), the apparent lack of competition between acetate metabolism and endogenous metabolism is puzzling. The present experiments provide no information concerning the mechanism which permits the two processes to go on side by side without interference. Approximately 33 % of the endogenous oxygen uptake is insensitive to fluoroacetate inhibition. This is roughly the amount of oxygen which would be required for the partial oxidation of carbohydrate to the level of acetate, the point at which the fluoroacetate block occurs.

Role of the endogenous metabolism. The constancy of the endogenous metabolism in the face of varying conditions, and its persistence during oxidation of exogenous nutrients, suggest that the endogenous pathways play a special role in Euglena metabolism which goes beyond merely providing a reserve of carbon and energy. It is worth noting, in this connexion, that *Euglena gracilis* will grow at maximum rates in the presence of substances which do not stimulate respiration appreciably above the endogenous level (Wilson, Buetow, Jahn & Levedahl, 1959). Thus, the rate of endogenous metabolism is sufficient to supply all the energy needed by growing cells. It is possible, therefore, that the endogenous metabolism is the direct source of energy for growth, movement, osmotic regulation, and other 'life processes', and that exogenous substrates are used only to replenish or increase the supply of endogenous reserves.

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The Fixation of Tetanus Toxin by Ganglioside

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SUMMARY

The fixation of tetanus toxin by brain ganglioside has been confirmed. A method has been developed for assaying the toxin-fixing capacity of ganglioside in the analytical ultracentrifuge. Under appropriate conditions ganglioside will fix many times its own weight of toxin. Ganglioside preparations from brain contain at least three gangliosides; at least two of these differ in their sialic acid contents and toxin-fixing capacities. The sialic acid residues (and their free carboxyl groups) are essential for toxin-fixation. Tetanus toxin does not appear (so far) to bring about any change in the ganglioside molecule.

INTRODUCTION

In a previous paper (Van Heyningen, 1959*b*) the tetanus toxin receptor in nervous tissue was tentatively identified as ganglioside. Brain ganglioside contains fatty acid (stearic acid), sphingosine, hexose (glucose and galactose), hexosamine (*N*-acetylgalactosamine) and sialic acid (*N*-acetylneuraminic acid) residues. The fatty acid, sphingosine and one of the hexose residues comprise a cerebroside moiety (Klenk & Lauenstein, 1953; Bogoch, 1958; Rosenberg & Chargaff, 1958). The presence of hydrophobic and hydrophilic groups can lead to micelle formation in aqueous solution, and the values for a molecular weight of about 250,000 reported by Folch, Arsove & Meath (1951), Bogoch (1958) and Rosenberg & Chargaff (1958) may be a reflexion of such micelle formation. Klenk & Gielen (1960) have recently reported that the ultracentrifugal behaviour of ganglioside in solution in dimethylformamide is consistent with a molecular weight of *c.* 1500. Their preparation of ganglioside appears to be a mixture of two gangliosides, for which they have suggested detailed structures. Each contains one moiety of cerebroside, one hexose residue and one sialic acid residue, and while one contains a hexosamine residue the other has an additional hexose residue instead. There is some uncertainty, however, about the sialic acid and hexosamine contents of brain ganglioside, and the reported values vary (Table 1).

Because of the formation of micelles in aqueous solution, conclusions about the homogeneity of preparations subjected to ultracentrifugation and electrophoresis are of questionable value. In the present work a ganglioside preparation, although electrophoretically and ultracentrifugally homogeneous, was found on investigation by chromatographic methods to contain a number of constituents. Some of these were eliminated, to leave a mixture of at least three gangliosides which were partially separated. In addition, a more refined method of assay of toxin-fixing

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capacity has been evolved. A study has been made of the effect of modifications in ganglioside on its capacity to fix toxin, and the important question of whether ganglioside is changed as a result of its interaction with toxin has been investigated.

Table 1. *Sialic acid and hexosamine contents of ganglioside preparations as variously reported*

	Residues per cerebroside residue	
Reference	Sialic acid	Hexosamine
Svennerholm (1956*a*, 1957*a*)	1·0	1·0
Klenk & Gielen (1960)	1·0 1·0	1·0 0
Folch, Meath & Bogoch (1956); see also Meltzer (1958)	1·5	0·67
Bogoch (1958)	1·5	1
Rosenberg & Chargaff (1958)	1·5	0·36

METHODS

Toxin. The tetanus toxin preparation, TD464D (Van Heyningen, 1959*a*), was kindly supplied by Dr C. G. Pope and Dr R. O. Thompson of the Wellcome Research Laboratories. It contains 40 % (w/w) protein (the rest is salt); 75 % of this protein is toxin.

Ganglioside. Fresh beef brain (8 kg.) was freed from blood and meninges under running tap water, minced in a Waring blendor with an equal volume of acetone, centrifuged, and the acetone extract discarded. The extraction was repeated twice to yield 1·3 kg. acetone-dried powder. This material was extracted for 5 hr. in a Soxhlet extractor with chloroform + methanol (1 + 2 vol.), to yield 380 g. extract (see Svennerholm, 1956*a*). Four 90 g. batches of extract were each dissolved in 3·5 l. chloroform + methanol (2 + 1 vol.) and the solutions dialysed for two days against running tapwater. During this time two phases formed within the dialysis tubes. The material in the upper aqueous phases from the four batches was concentrated in a rotating evaporator and freeze-dried to yield 14 g. crude ganglioside containing 0·3 μmole sialic acid/mg. (see Folch *et al.* 1951). Of this crude ganglioside 10 g. were dissolved in 400 ml. chloroform + methanol (2 + 1 vol.) containing 10 % (w/v) $CaCl_2.H_2O$. After standing for some hours at room temperature the deposit was filtered off (see Rosenberg & Chargaff, 1958), the filtrate dialysed against running tap water for two days, and the ganglioside recovered from the upper phase as before; the yield was 4·1 g. crude ganglioside containing 0·6 μmole sialic acid/mg. This material was again dissolved in chloroform + methanol, treated with calcium chloride, and recovered by partition-dialysis to yield 3·0 g. ganglioside containing 0·82 μmole sialic acid/mg. This was the calcium salt of ganglioside. To convert it to the free acid 3 g. were dissolved in 80 ml. water and the solution brought to pH 1·4 with 12 N-HCl. The acidic solution was dialysed in the cold with agitation against several changes of 1 l. of distilled water until the pH value was constant at 3·0 (Folch *et al.* 1951). The final yield was 2·7 g. of partially purified ganglioside containing 0·73 μmole sialic acid/mg., 0·31 μmole hexosamine/mg. This material was ninhydrin-negative, and contained less than 0·4 % protein according to the method of Lowry *et al.* (1951). It showed a single peak on electrophoresis (Fig. 1 (ii)) and ultracentrifugation (Fig. 2 (i)), but on paper chromatography (Fig. 6*c*) several substances were detected. Spots 4 and 5 were much fainter than spots 1, 2 and 3. Spots 3, 4 and 5 were non-metachromatic and stained blue-mauve, spots 1 and 2 were metachromatic and stained pink-mauve.

Column chromatography. Mallinckrodt's Analytical Reagent Silicic Acid 100 mesh (Savory and Moore, London) was ground to 300 mesh and treated according to Hirsch & Ahrens (1958). Silicic acid (10 g.) was suspended in 35 ml. chloroform, poured into tubes, 1·5 cm. diameter, 25 cm. long, and washed with a further 50 ml. chloroform. Ganglioside was dissolved in 6 % (v/v) methanol in chloroform and loaded on the column, at not more than 25 mg./g. sialic acid. Elution was performed with methanol + chloroform mixtures of stepwise increasing methanol concentration as indicated below. The chloroform used was washed with water, dried with $CaCl_2$ and distilled before use; methanol was distilled before use.

Paper chromatography. Schleicher & Schull 2045B smooth paper (Gallenkamp-Towers, London) was used, in strips 20 cm. long and 11 cm. wide, after washing overnight with 5 % (v/v) acetic acid by descending chromatography, followed by washing with water and air drying. Samples (200 μg.) dissolved in 10 μl. water were placed on the papers in 1 cm. streaks and the chromatograms developed by the ascending technique with freshly prepared mixtures of di-iso-butylketone, acetic acid, H_2O (40:30:7, v/v; Marinetti & Stotz, 1956), in tightly closed cylindrical jars, 21 cm. high, 12·5 cm. diameter. After overnight running the papers were air dried, stained by immersion in 0·02 % (w/v) cresyl violet in 1 % (v/v) acetic acid in water and heating at 60° for 10 min., and washed with 1 % (v/v) acetic acid. This dye stains lipids, acid lipids showing metachromasy, particularly in wet papers.

Analyses. Sialic acid was determined by the resorcinol method of Svennerholm (1957*b*), with *N*-acetylneuraminic acid (kindly supplied by Professor G. Blix and Dr L. Svennerholm) as a standard. Hexosamine was determined by the Procedure A of Svennerholm (1956*b*), with glucosamine hydrochloride as a standard.

Ultracentrifugal studies. The Spinco Model E Analytical Ultracentrifuge, with the RTIC unit and a phase plate in the schlieren-optical system, was used. Toxin and ganglioside were dissolved in 0·1 M-phosphate buffer (pH 7·0). The temperature of the rotor varied in different runs from 18° to 21°. Runs were carried out at 59,780 rev./min. for 23 min. in single sector cells and at 42,040 rev./min. for 60 min. in double sector cells. Areas under the peaks of the schlieren diagrams (phase plate angle 60°) on the photographic plates were measured by projecting the diagrams in a photographic enlarger with an enlargement of 10 diameters, tracing the diagrams on paper, drawing a midline through the thickness of the phase-plate lines, and measuring the areas so enclosed by means of a polar planimeter with an accuracy of $\pm 0{\cdot}5$ %. The schlieren diagrams shown in this paper are derived from these tracings.

Electrophoresis. The Antweiler microelectrophoresis apparatus, with schlieren optical attachment (Boskamp; Shandon, London), was used. Samples were dissolved in 0·05 M-phosphate buffer (pH 7·0) and dialysed against the same buffer and subjected to electrophoresis at 75 v. for 10 min.

RESULTS

Studies on the combination of toxin and ganglioside

Electrophoretic studies. In previous studies (Van Heyningen, 1959*b*) the reaction between the water-soluble tetanus toxin and water-soluble ganglioside was demonstrated in the preparative (Spinco Model L) ultracentrifuge by determining the effect of the more rapidly sedimenting ganglioside on the rate of migration of the toxin from the upper to the lower half of the centrifuge tube. This was done by measuring the protein contents of the upper and lower halves of centrifuge tubes after centrifuging the toxin at 40,000 rev./min. for 1 hr. in presence and absence of ganglioside. The combination of toxin and ganglioside can also be demonstrated by

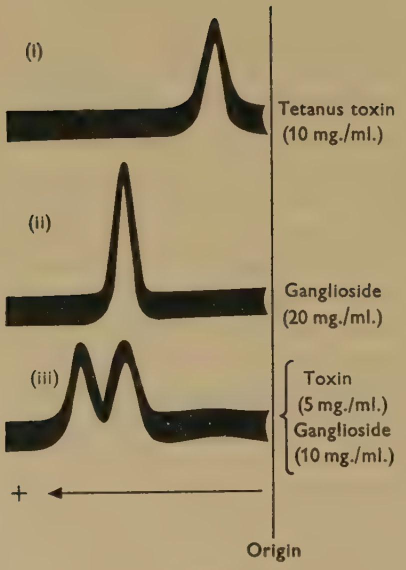

Fig. 1

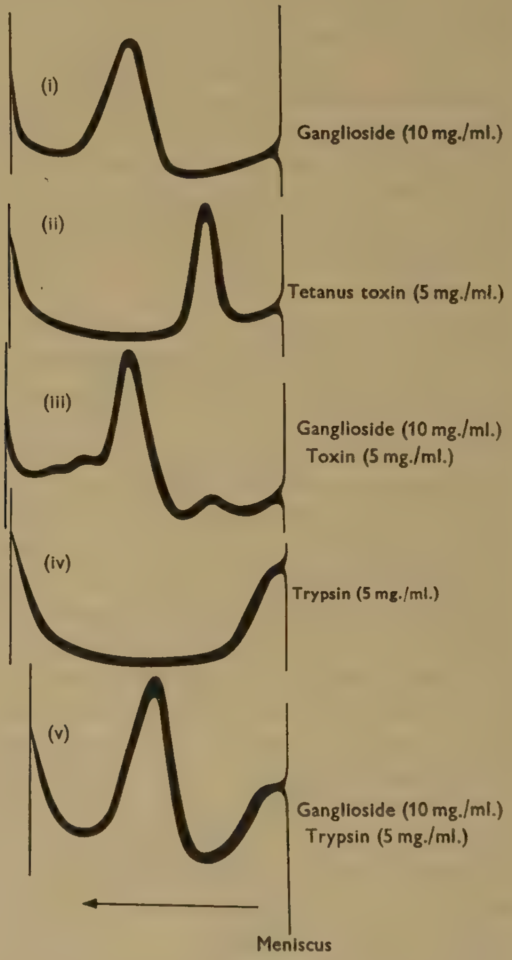

Fig. 2

Fig. 1. Fixation of tetanus toxin by ganglioside demonstrated by boundary electrophoresis.

Fig. 2. Fixation of tetanus toxin, and non-fixation of trypsin, demonstrated in the analytical ultracentrifuge.

electrophoresis. Fig. 1 (i) shows that toxin alone migrated towards the positive pole much more slowly than ganglioside (Fig. 1 (ii)). Fig. 1 (iii) shows that when toxin and ganglioside were mixed the more slowly moving toxin peak was absent and instead a complex of peaks appeared, moving at about the same rate as ganglioside.

Ultracentrifugal studies; qualitative. The combination of toxin and ganglioside can also be demonstrated in the analytical ultracentrifuge. Fig. 2 (i) shows that ganglioside alone sedimented faster than toxin alone (Fig. 2 (ii)). Fig. 2 (iii) shows the sedimentation diagram of a mixture of toxin and ganglioside. The slowly moving toxin peak has almost disappeared (see section *quantitative* below), and we now have a complex of peaks sedimenting as fast as, and faster than the ganglioside peak, since complexes of greater molecular weight than ganglioside alone are formed. Fig. 2 (iv) shows that under the same conditions of centrifugation the peak of trypsin, a protein which does not combine with ganglioside at the buffer concentration used (see van Heyningen 1959*a*), did not move completely away from the meniscus. Fig. 2 (v) shows that the sedimentation of trypsin, unlike that of tetanus toxin, was unaffected by the presence of ganglioside.

The following substances were found not to fix tetanus toxin. (1) Sialic acid-free ganglioside, prepared by acid hydrolysis of brain ganglioside and kindly supplied by Professor E. Klenk. (2) Brain ganglioside in which the carboxyl groups of the sialic acid residues had been methylated with diazomethane. When this substance was saponified (in 0·1 N-NaOH for 2 hr. at 37°) it fixed toxin again. (3) Sialic acid alone (the preparative ultracentrifugal technique was used for this demonstration; although 40 % of the toxin in the upper half of a centrifuge tube migrated to the lower half during spinning for 2 hr. at 40,000 rev./min., no measurable amount of sialic acid migrated in the presence or absence of toxin under the same conditions). (4) Ovine mucoid substance with a sialic acid content of *c.* 0·5 μmole/mg. and a molecular weight of *c.* 40,000, kindly supplied by Professor Gunnar Blix. (5) Hexosamine-free ganglioside from horse red blood cells (Klenk & Lauenstein, 1953), kindly supplied by Professor E. Klenk.

Ultracentrifugal studies; quantitative. The qualitative demonstration of the fixation of toxin by ganglioside in the analytical ultracentrifuge led to the development of a quantitative assay of toxin-fixing capacity. This involved centrifuging a mixture of toxin and ganglioside and determining the concentration of toxin not fixed, by measuring the area of the toxin peak in the schlieren diagram. This area is a linear function of the concentration of toxin. These measurements require that the baselines of the peaks should be accurately delineated, and for this purpose double-sector cells were used, with buffer in one sector (to give the base lines) and the solution of toxin and ganglioside in buffer in the other (to give the peaks). Double sector cells cannot be spun at speeds greater than 42,040 rev./min. Fig. 3*a* shows the series of schlieren diagrams obtained when constant concentrations of 5 mg. toxin protein/ml. were mixed with concentrations of ganglioside increasing from 0–10 mg./ml. With increasing ganglioside concentration the area of toxin peak progressively decreased as more toxin was sedimented with the ganglioside. At concentrations of 2 mg. ganglioside/ml. and above there was no further decrease in the area of the toxin peak and a peak of constant size remained, corresponding in area to 25 % of the original peak. This residual peak is due to the 25 % of non-toxic protein in the toxin preparation (75 % pure, see Methods) which is not fixed by the ganglioside.

It is interesting to see in these diagrams that the rapidly sedimenting ganglioside-toxin peaks are complex, representing several ganglioside-toxin combinations, and that the smaller the concentration of ganglioside the greater the speed with which the complexes sedimented. (Control experiments showed that the rate of sedimentation of ganglioside alone was independent of concentration.) Presumably at high ratios of toxin to ganglioside more toxin is fixed per unit weight of ganglioside and consequently the weight of the resultant particle is greater.

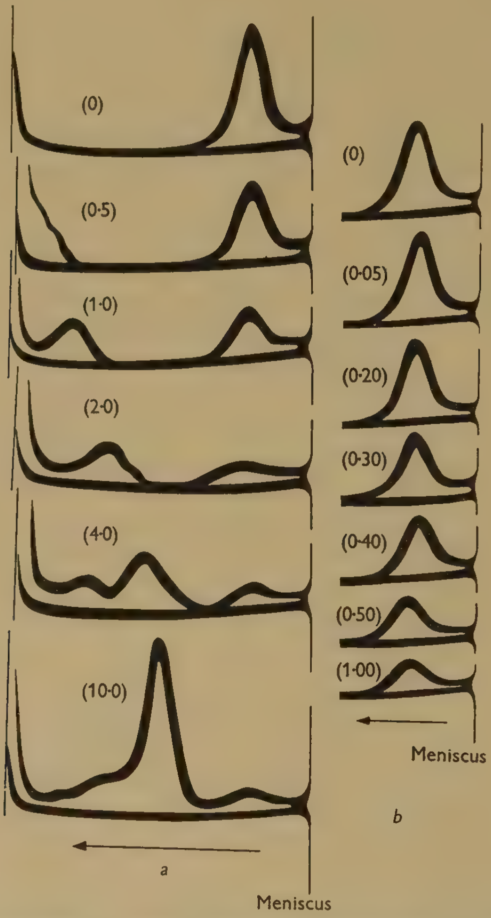

Fig. 3. Quantitative assay of toxin fixation by ganglioside in the analytical ultracentrifuge. Toxin protein concentration 5 mg./ml. in all cases, ganglioside concentration (mg./ml.) shown in brackets. *a*, Wide range of ganglioside concentrations; *b*, toxin protein peaks after treatment with smaller range of ganglioside concentrations.

To provide a quantitative assay of toxin-fixing capacity, a smaller range of ganglioside concentrations was mixed with constant concentrations of 5 mg. toxin protein/ml. Fig. 3*b* shows the peaks of the unfixed protein during centrifugation. From the areas of these peaks the amounts of unfixed protein, and thus of fixed protein, were calculated. (In 10 separate experiments the values obtained for the area due to 5 mg. toxin protein, including pipetting errors, were 31·6, 34·5, 32·4, 29·8, 33·8, 32·8, 32·7, 32·3, 31·2, 31·4; mean 32·3 mm.^{2}). The points in Fig. 4 show the relation between amount of ganglioside and amount of toxin fixed. Evidently the curve through these points is very steep at low concentrations of ganglioside. Where the data are replotted to show weight of toxin fixed per unit weight of ganglioside (on the ordinate) as a function of weight of toxin fixed per unit volume, the intercept with the ordinate (i.e. infinite ratio of unfixed toxin to ganglioside) corresponds to a value of 23·5 mg. toxin fixed/mg. ganglioside (or 1 mole toxin/2 mole ganglioside). The (unbroken) curve in Fig. 4 is the Standard Curve obtained by replotting the smooth (broken) curve drawn through the points in this figure, with mg. toxin fixed on the ordinate and Receptor Units on the abscissa. The Receptor Unit (RU) is defined as the amount of ganglioside that fixes 20 μg. tetanus toxin (= 10L+ units toxin at 2 μg. toxin/L+ unit; see van Heyningen, 1959*a*) at an infinite ratio of toxin to ganglioside. (The RU now defined differs to an unknown degree from the RU previously defined under different conditions for the insoluble ganglioside + cerebroside receptor complex; van Heyningen, 1959*a*). The steps in determining the RU content of a ganglioside preparation are now as follows. To one 0·25 ml. volume containing 25 mg. tetanus toxin preparation TD 464 D (i.e. 10 mg. toxin protein)/ml. 0·1 M-phosphate buffer (pH 7) is added 0·25 ml. buffer, and to another 0·25 ml. volume of toxin solution is added 0·25 ml. of a ganglioside solution of appropriate concentration in buffer. A portion (0·4 ml.) of the solution containing toxin only is placed in one sector of the double sector wedge cell, and 0·4 ml. of the ganglioside + toxin solution is placed in one sector of the double sector standard cell. Buffer (0·4 ml.) is placed in the remaining sectors of each cell. The cells are then spun at 42,040 rev./min. and after 1 hr. the schlieren diagrams are photographed, and the areas of the protein peaks measured. From the decrease in area due to the presence of ganglioside the mg. protein fixed is calculated and from this value the RU content of the ganglioside preparation is derived from the Standard Curve (Fig. 4).

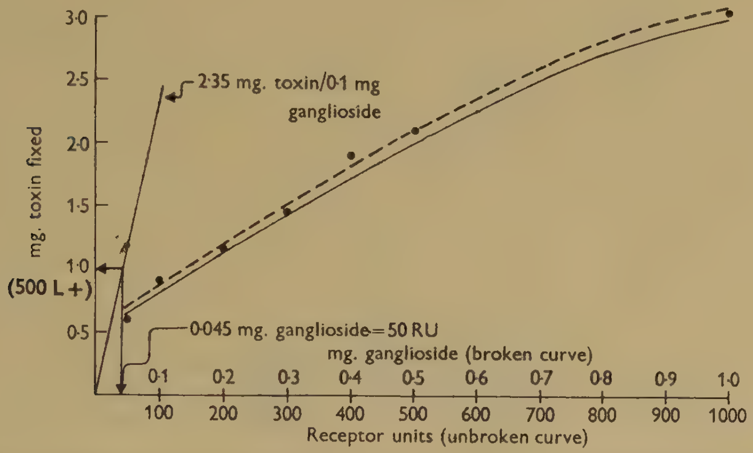

Fig. 4. Relation between weight of toxin fixed and weight of ganglioside used (broken curve), and the Standard Curve (unbroken) derived from these data.

Purification of ganglioside

The partially purified sample of ganglioside (0·73 μmole sialic acid, 0·31 μmole hexosamine, 1100 RU/mg.; see Methods) was further purified by chromatography on a column of silicic acid by a procedure recommended by Dr L. Svennerholm. Ganglioside (200 mg.) was dissolved in a minimum volume of 6 % (v/v) methanol in chloroform, and the column successively eluted with 50 ml. 10 %, 50 ml. 25 % and 150 ml. 50 % (v/v) methanol in chloroform. The eluate was collected in 10 ml. volumes and the sialic acid content determined. The ganglioside all emerged in the 50 % (v/v) methanol eluate (Fig. 5). Evidently it was still inhomogeneous with respect to sialic acid content and toxin-fixing capacity, although the hexosamine content of the material in all the tubes appeared to be constant (Table 2). On paper chromatography the pooled material from the 50 % methanol eluate showed spots in positions 1, 2 and 3, and spots 4 and 5 were absent (compare Fig. 6*c*). This material was placed on a fresh column of silicic acid, and after elution with 50 ml. 10 % (v/v) methanol in chloroform and 50 ml. 25 % methanol it was eluted with 30 % methanol likewise in chloroform. Ganglioside first emerged in the 30 % methanol eluate and the column was eluted with this solvent (*c*. 200 ml.) until only negligible quantities of sialic acid-containing material emerged. Seventy-five mg. of material, which we shall call 'fast' ganglioside, was recovered in the 30 % methanol eluate. The column

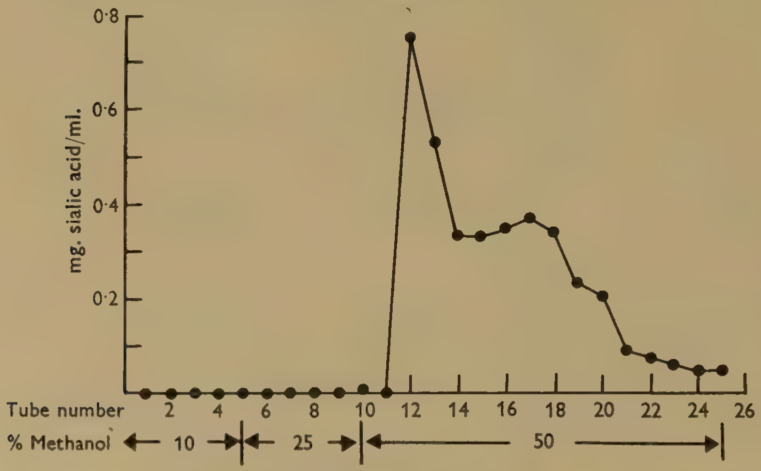

Fig. 5. Inhomogeneity of ganglioside emerging in 50 % methanol in chloroform eluate from silicic acid column.

Table 2. *Data on ganglioside preparations eluted from a silicic acid column with* 50 % (*v*/*v*) *methanol in chloroform* (*see Fig.* 5)

Pooled tubes	Hexosamine (μmole/mg.)	Sialic acid (μmole/mg.)	RU/mg.	RU/μmole sialic acid
12, 13	0·27	0·77	950	1234
14, 15	0·26	0·83	1090	1314
16–25	0·28	0·88	1170	1330

was then eluted with 200 ml. 50 % methanol, and 90 mg. 'slow' ganglioside was recovered. Diagrams of the paper chromatograms of the 'fast' and 'slow' preparations are shown in Fig. 6, *a* and *b*. The 'fast' preparations consist mainly of the non-metachromatic material (spot 3), and the 'slow' mainly of metachromatic material (spot 1). Both preparations contained a proportion of the metachromatic component 2. The metachromatic and non-metachromatic materials were eluted from the paper and rechromatographed, separately and together, and no interconversion was observed. Both 'slow' and 'fast' preparations have the same hexosamine content (0·31 μmole/mg. in 'slow', 0·30 μmole/mg. in 'fast'), but the 'slow' ganglioside contains more sialic acid (0·93 μmole/mg.) and has a greater toxin-fixing capacity (1310 RU/mg.) than the 'fast' (0·75 μmole sialic acid/mg., 950 RU/mg.). We have observed similar differences in chromatographic properties, sialic acid content and toxin-fixing capacities between ganglioside preparations of other workers. Thus a mucolipid preparation provided by Dr Erwin Chargaff was mainly 'slow' and metachromatic on paper chromatography and contained 0·89 μmole sialic/mg and 1190 RU/mg., whereas a ganglioside preparation provided by Professor E. Klenk was mainly 'fast' and non-metachromatic and contained 0·66 μmole sialic acid/mg.

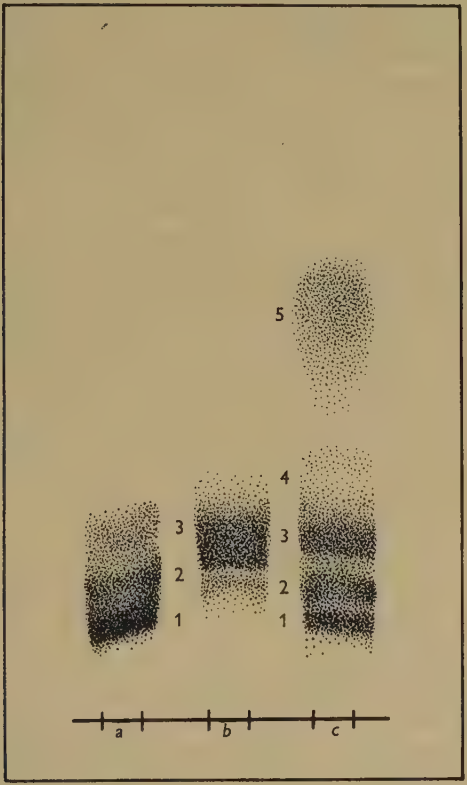

Fig. 6. Paper chromatograms of 'slow' (*a*), 'fast' (*b*), and partially purified (*c*), preparations of ganglioside. Developed by ascending technique in di-*iso*butyl ketone, acetic acid, water and stained with cresyl violet. Spots 1 and 2 metachromatic (pink-mauve) 3, 4 and 5 non-metachromatic (blue-mauve).

and 540 RU/mg. Since sialic acid is split off when ganglioside is heated at 100° at pH 3, and possibly on prolonged standing in solution at room temperature (e.g. see Bogoch, 1958), we wondered whether the 'fast' ganglioside is a degradation product derived from the 'slow' by loss of sialic acid during preparation, or whether they both exist in nervous tissue. We were not able to change the relative proportions of 'fast' and 'slow' in ganglioside preparations by the following variations in procedure: (*a*) plunging chopped-up beef brain in dry ice-cooled acetone immediately after killing, as compared with standing brain at room temperature overnight before acetone-dehydration; (*b*) extracting dehydrated brain with chloroform-methanol at room temperature as compared with extraction in a Soxhlet apparatus; (*c*) deionizing by treatment with Amberlite CG 50 as compared with dialysis at pH 1·4–3.

Is ganglioside changed by interaction with tetanus toxin?

We examined the question whether ganglioside is changed as a result of its interaction with tetanus toxin. Ganglioside (5 mg./ml.) and tetanus toxin (10 μg., or 75,000 LD 50/ml.) were incubated together in 0·1 M-phosphate buffer (pH 7) for 5 hr. at 37° and the solution then dialysed overnight at 2° with constant agitation against an equal volume of water. The diffusate was retained and dialysis of the residue continued for 5 hr. against running tap water. Tests for free amino groups, sialic acid and hexosamine were then carried out on the residue and the diffusate. The concentration of ganglioside was such that a 10 % liberation of amino groups, or of hexosamine or sialic acid in a diffusible form, would have been detectable. The only positive reactions observed were those for sialic acid and hexosamine on the non-diffusible residue, which suggests that the amino groups of the hexosamine, sialic acid and sphingosine residues were not liberated, and that there was no liberation of diffusible material containing hexosamine or sialic acid. Bangham & Dawson (1959) showed that lecithin was not attacked by lecithinase unless the positive charge was changed to a negative charge by the addition of various negatively charged substances at threshold concentration. Positively charged cations or amphiphatic molecules reversed the effect. With the thought that similar considerations of charge (either negative or positive) might apply to the possible enzymic cleavage of ganglioside, the experiments above were repeated in the presence of 0·02 M-$CaCl_2$ (in which case collidine acetate buffer, pH 6·8, was used), and cetyltrimethylammonium bromide, sodium dodecyl sulphate, dicetylphosphoric acid, and saponin, all at 0·25 mg./ml. None of these substances brought about any positive results. Since ganglioside occurs in extracts of nervous tissue in the form of a complex with cerebroside (van Heyningen, 1959*b*) the same tests were carried out with ganglioside cerebroside complex, again with negative results.

Ganglioside (10 mg./ml. 0·05 M-phosphate buffer, pH 7) was incubated overnight at 37° in the presence and absence of tetanus toxin (10 μg./ml., 75,000 LD 50/ml.); the two preparations behaved identically in the electrophoresis apparatus and in the analytical ultracentrifuge.

If tetanus toxin were an enzyme with ganglioside as its substrate, one would not expect the ganglioside to fix the toxin after the toxin had catalysed a change in it. This point was tested in the following way. An insoluble ganglioside + calcium + cerebroside complex was made (Van Heyningen, 1959*b*) and suspended in water to give a concentration of 1 mg. ganglioside/ml. To samples of this suspension were added equal volumes of 0·133 M-phosphate buffer (pH 7) or a solution containing 1 mg. (= 7 million LD 50) toxin/ml. 0·133 M-phosphate buffer (pH 7). These two mixtures were incubated for 16 hr. at 37°, spun at 5000 rev./min., the pellets washed with water, and the adsorbed protein removed with 0·1 N-NaOH. The ganglioside + calcium + cerebroside complexes which had thus been treated with toxin overnight and deproteinized with alkali, and the control preparation that had not been treated with toxin, were resuspended to the same concentration as before and again added to equal volumes of toxin solution and the amount of adsorbed protein measured in the manner previously described (van Heyningen, 1959*a*). The receptor complex which had been treated with toxin adsorbed 88 μg. protein/mg. on the second suspension in toxin solution, and the control preparation 81 μg. protein/mg. It appeared therefore that the toxin-fixing capacity of the ganglioside + cerebroside complex was unaffected by treatment overnight with toxin at a concentration of 3·5 million LD 50/ml.

We also tested the possibility that complement might be fixed during the toxin + ganglioside interaction, but found that guinea pig complement (2 MHD) still haemolysed sensitized sheep red cells after incubation with toxin + ganglioside mixtures. The toxin concentration ranged from 0·11 mg./ml. (800,000 LD 50/ml.) to 0·000,000,4 mg./ml. (3 LD 50/ml.) and the ganglioside concentration from 0·4 mg./ml. to 0·05 mg./ml. The reagents were added in the order complement, toxin, ganglioside, 1 hr. at 37°, haemolytic system, 20 min. at 37°.

DISCUSSION

In our most purified preparations of ganglioside at least three components have been observed on paper chromatograms, but on silicic acid columns only two were distinguished, and the absence of the third component was probably due to inadequate resolution. The three components have not been sufficiently separated from each other to merit a complete chemical analysis. The presence of adsorbed proteins might account for inhomogeneity on columns, but our preparations contain no proteins, and we believe we are dealing with at least three different gangliosides, and that irrelevant impurities have been removed. The 'fast' and 'slow' fractions separating on the silicic acid columns have the same hexosamine content, but differ in rate of movement and staining with cresyl violet on paper chromatography, in sialic acid content, and in toxin-fixing capacity. They contain the third, metachromatic, component in common.

The reported sialic acid contents of ganglioside vary (Table 1). Comparison of our preparations with those of others suggests that the preparation of Klenk & Gielen (1960), which is a mixture of two gangliosides each containing 0·66 μmole sialic acid/mg., is 'fast' and non-metachromatic, whereas the preparation of Rosenberg & Chargaff (1958), which contains more sialic acid (0·89 μmole/mg.) is 'slow' and metachromatic. We are not in a position to say whether the 'fast' and 'slow' gangliosides exist as such in nervous tissue, or whether the 'fast' is derived from the 'slow' by loss of some sialic acid during extraction and purification.

The fixation of tetanus toxin is dependent on the presence of the sialic acid residue in ganglioside, and, at least within the range provided by the 'fast' and 'slow' preparations, roughly proportional to the sialic acid content. The free carboxyl group of the sialic acid residue is essential for toxin-fixation.

The hexosamine-free ganglioside from horse red blood did not fix toxin, but this may be due to factors other than the absence of hexosamine, e.g. *N*-glycolyl rather than *N*-acetyl substituents in the sialic acid residues, differences in fatty acid residues, etc.

If tetanus toxin brings about a change in ganglioside it is by no means obvious. All the tests that we were able to devise to detect any change were negative and therefore inconclusive.

We are grateful to Dr A. Woodin for helpfully critical discussions; to Forskare L. Svennerholm for receiving one of us (W. E. van H.) in his laboratory and giving him invaluable advice on column and paper chromatography of gangliosides, as well as a sample of pure sialic acid; to Dr G. G. Pope and Dr R. O. Thompson for the tetanus toxin; to Professor G. Blix for samples of sialic acid and mucoid substances; to Professor E. Klenk for various ganglioside preparations; to Dr E. Chargaff for a sample of mucolipid; to the Oxford and District-Co-operative Society Ltd. for the supply of beef brain; to Mr J. Otway for technical assistance and to Mrs Galatea Collins for non-technical assistance. Very special thanks are due to the Wellcome Trustees for the most generous grant to W. E. van H. of the Spinco Analytical Ultracentrifuge and the Antweiler Microelectrophoresis apparatus. W. E. van H. also acknowledges with gratitude that this work was assisted in part by a grant from the Office of Naval Research of the United States Department of the Navy (Project No. 103–474). P. A. M. was the John Simon Guggenheim Memorial Fellow.

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The Relation between the Tetanus Toxin-Fixing and Influenza Virus-Inhibiting Properties of Ganglioside

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(*Received* 25 *July* 1960)

SUMMARY

The capacity of a number of gangliosides to fix tetanus toxin and to inhibit haemagglutination by influenza virus was investigated. Toxin fixation increased with total sialic acid content but not in strict proportion. Whether or not the sialic acid is bound by neuraminidase-labile linkage appears to be irrelevant in determining whether toxin is fixed. Viral inhibition by ganglioside also is related to the total content of sialic acid; but there exists at least one ganglioside which has very little capacity to react with virus and whose sialic acid is virtually all insensitive to neuraminidase. Tetanus toxin does not prevent neuraminidase from attacking ganglioside.

INTRODUCTION

Ganglioside from nervous tissue has been shown to act as a specific receptor for tetanus toxin (Van Heyningen, 1959) and the sialic acid residue(s) of this compound has been shown to be essential for toxin-fixation (Van Heyningen & Miller, 1961). It is well known that sialic acid-containing substances also act as influenza virus receptors. Rosenberg, Howe & Chargaff (1956) found that their preparation of mucolipid, which contained a polypeptide moiety in addition to ganglioside, inhibited influenza virus, and was susceptible to viral and cholera neuraminidase, whereas samples of ganglioside which did not contain amino acid residues were inactive. They considered that the polypeptide moiety, in addition to the sialic acid residue, was essential for viral inhibitory activity. It has been observed that amino acids are present in ganglioside preparations extracted from wet brain, but not in preparations extracted from acetone-dehydrated brain (Folch, Arsove & Meath, 1951; Svennerholm, 1956). Bogoch (1957) showed that a preparation of brain ganglioside from wet brain inhibited viral haemagglutination. Kuhn (1958) separated two fractions of brain ganglioside from dehydrated brain on a cellulose column eluted with methanol + pyridine + water and found that the more rapidly moving fraction (G 2), containing 21 % sialic acid, did not inhibit viral haemagglutination and was not susceptible to *Vibrio cholerae* neuraminidase; the more slowly moving fraction (G 0·5) containing 31 % sialic acid, inhibited viral haemagglutination and was susceptible to neuraminidase. Van Heyningen & Miller (1961) separated brain ganglioside (prepared from dehydrated brain and containing no amino acid residues) into 'fast' and 'slow' fractions from silicic acid columns eluted with methanol + chloroform. The 'fast' preparation contained 0·75 μmole sialic acid/mg. and the 'slow' 0·93 μmole/mg. The tetanus toxin-fixing capacities of these fractions were approximately proportional to their sialic acid contents. They also showed that the mucolipid preparation of Rosenberg *et al.* (1956) was 'slow' and contained 0·89 μmole sialic acid/mg., whereas an amino acid-free preparation supplied by Professor E. Klenk was 'fast' and contained 0·66 μmole sialic acid/mg. The tetanus toxin-fixing capacities of these preparations were also roughly proportional to their sialic acid contents. We have now investigated the influenza virus-inhibiting properties of these various ganglioside preparations in relation to their toxin-fixing capacities.

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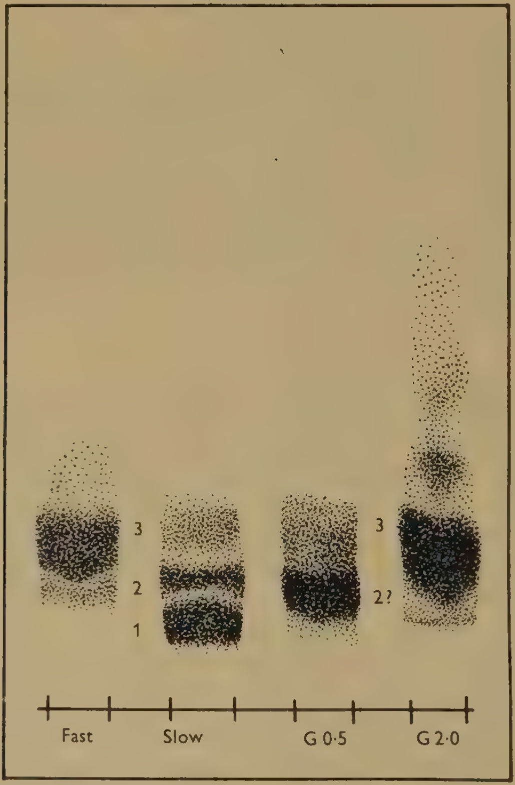

Fig. 1. Paper chromatograms of various ganglioside preparations. Components 1 and 2 metachromatic, 3 non-metachromatic.

METHODS

Ganglioside preparations. The preparation (or source) and properties of the 'partially purified', 'fast', 'slow', 'Chargaff' and 'Klenk' gangliosides are described in the preceding paper (Van Heyningen & Miller, 1961). The G 2·0 and G 0·5 preparations were kindly supplied by Professor Richard Kuhn. Their paper chromatographic behaviour in comparison with 'fast' and 'slow' ganglioside preparations is shown in Fig. 1.

Paper chromatography. As described by Van Heyningen & Miller (1961), except that for Fig. 3 the ratio of di-*iso*butylketone, acetic acid, water was 40:25:5, rather than 40:30:7.

Assays. Total sialic acid was estimated by the resorcinol method of Svennerholm (1957); free sialic acid by the thiobarbituric acid assay of Warren (1959); tetanus-toxin fixing capacity (RU/mg.) by the method of Van Heyningen & Miller (1961); influenza virus-inhibiting capacity essentially by the method of Howe (1951). The PR8 strain of influenza virus was harvested from eggs, the combined allantoic fluid centrifuged and the sedimented virus resuspended in 0·1 vol. 0·15 M-NaCl. Virus prepared in this way (haemagglutinin titre 64,000) was kindly supplied by Dr A. Isaacs. Indicator virus was prepared by heating this preparation for 30 min. at 55–56°. The titrations were carried out in MRC pattern Perspex plates. Serial two-fold dilutions of ganglioside contained in 0·2 ml. 0·15 M-NaCl, buffered to pH 7·2 with 0·01 M-phosphate, were mixed with 1–2 haemagglutinin units of indicator virus and allowed to stand in the refrigerator for 30 min.; 0·1 ml. of a 2 % suspension of washed chicken erythrocytes was then added, and after standing 1–2 hr. in the cold, the patterns of the sedimented erythrocytes were observed. The end-point used was the smallest amount of ganglioside giving just short of complete inhibition of haemagglutination.

Neuraminidase. Two preparations were used: (*a*) crude freeze-dried *Vibrio cholerae* filtrate (N. V. Philips-Roxane, Amsterdam), (*b*) crystalline *V. cholerae* neuraminidase kindly supplied by Dr G. L. Ada. Neuraminidase (RDE) activity was estimated by the method of Burnet & Stone (1947). The Ca + acetate + saline used in experiments with neuraminidase contained per l. distilled water: 12·38 g. sodium acetate, 5·0 g. NaCl, 1·0 g. $CaCl_2.H_2O$; adjusted to pH 6·2 with 2 N-acetic acid.

RESULTS

Inhibition of haemagglutination

The viral haemagglutination-inhibiting property of the various ganglioside preparations is shown in Table 1.

Action of neuraminidase on ganglioside

The thiobarbituric acid assay (Warren, 1959) is useful for the measurement of enzymic liberation of sialic acid from ganglioside since it estimates only free sialic acid. Figure 2*a* shows the time course of the liberation of sialic acid from 'slow' ganglioside by crude *Vibrio cholerae* neuraminidase (75 units/ml.). Maximal splitting was reached in about 8 hr. Figure 2*b* shows the liberation of sialic acid from 'slow' ganglioside by increasing concentrations of crystalline neuraminidase in 16 hr. Maximal splitting was obtained with 75–100 enzyme units/ml.

Comparative susceptibility of various ganglioside preparations to neuraminidase. Neuraminidase-labile sialic acid of the several ganglioside preparations was estimated by incubating 160 μg. ganglioside with 4000 units of crystalline neuraminidase in a volume of 0·4 ml., pH 6·2, at 36°. After 16 hr. free sialic acid was measured (Warren, 1959) and the values obtained corrected for small amounts of colour and turbidity given by untreated ganglioside. The results are shown in Table 1.

Table 1. *Chromatographic behaviour, neuraminidase-sensitivity, viral-inhibition and toxin-fixation of ganglioside preparations*

Ganglioside preparation	Chromatographic properties of main component(s)		Total sialic acid (μmole/mg.)	Fraction of total sialic acid liberated by neuraminidase (%)	Amount required to inhibit 1–2 haemagglutinin units of influenza virus (μg.)	Toxin-fixing capacity	
	Movement	Staining with cresyl violet				RU/mg.	RU/μmole sialic acid
Partially purified	Fast and slow	N and M*	0·73	44	8	1100	1505
Fast	Fast	N	0·75	35	8	950	1280
Slow	Slow	M	0·93	57	1	1310	1400
Kuhn G0·5	Slow	M	0·82	33	0·5	820	1000
Kuhn G2·0	Fast	N	0·58	2	20	580	1000
Chargaff mucolipid	Slow	M	0·89	47	0·5	1190	1340
Klenk ganglioside	Fast	N	0·66	21	10	540	820

*M = metachromatic, i.e. stains pink-mauve; N = non-metachromatic, i.e. stains blue-mauve.

A preparation of influenza virus, 50 times as concentrated (the greatest practicable) as the allantoic fluid from which it was derived, released 48 % of the total sialic acid of the 'slow' ganglioside in 16 hr. at 36° (compare 57 % released by *Vibrio cholerae* neuraminidase).

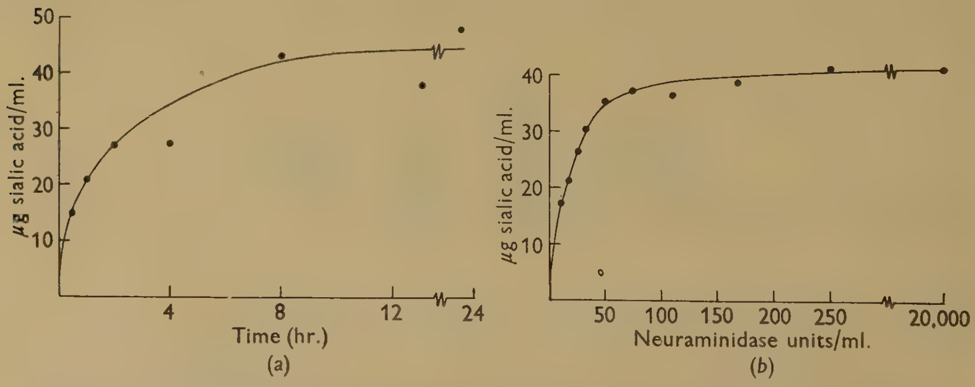

Fig. 2*a*. Course of appearance of sialic acid in a mixture of ganglioside and *Vibrio cholerae* filtrate. One ml. crude *V. cholerae* filtrate reconstituted to twice its original volume with calcium + acetate + saline (pH 6·2) was added to one ml. of the same diluent containing 0·8 ml. 'slow' ganglioside. During incubation at 36°, 0·2 ml. samples were removed at the times indicated and delivered into tubes containing 0·1 ml. sodium periodate in phosphoric acid, and free sialic acid subsequently estimated. The values are corrected for colour given by the *V. cholerae* filtrate.

Fig. 2*b*. Liberation of sialic acid as a function of enzyme concentration. Each point represents the free sialic acid found after incubating for 16 hr. at 36° 80 μg. 'slow' ganglioside in 0·1 ml. calcium + acetate + saline (containing 0·1 % gelatin) + 0·1 ml. crystalline *V. cholerae* neuraminidase in same diluent. The values are corrected for the small amount of colour given by the ganglioside alone.

Fixation of tetanus toxin

The tetanus toxin-fixing capacities of the various ganglioside preparations are shown in Table 1. It is evident that even when practically none of the sialic acid was neuraminidase-sensitive (as in preparation G 2·0) ganglioside still fixed toxin. The data suggest that neuraminidase-insensitive as well as neuraminidase-sensitive sialic acid is concerned in toxin-fixation. In order further to test this point a solution containing 1·2 mg. 'slow' ganglioside and 6000 units neuraminidase in 3 ml. Ca + acetate + saline was incubated for 16 hr., with liberation of 48 % of the total sialic acid. The mixture was dialysed overnight against running tap water and freeze-dried. The product was found to contain 580 RU/mg., or 1140 RU/μmole sialic acid.

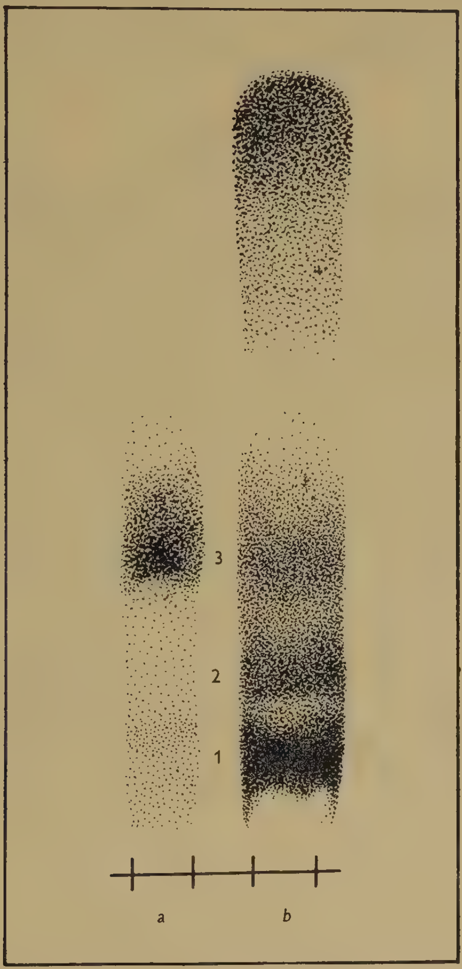

Fig. 3. Paper chromatogram of 'slow' ganglioside after treatment with neuraminidase (*a*) compared with partially purified ganglioside (*b*). Components 1 and 2 metachromatic, 3 non-metachromatic.

Figure 3 shows a paper chromatogram of the neuraminidase-treated preparation, compared with unfractionated ganglioside. It can be seen that the neuraminidase had converted 'slow' ganglioside into 'fast' ganglioside.

The possibility was tested that tetanus toxin might competitively inhibit the action of neuraminidase on ganglioside, but as much as 2·6 mg. toxin/ml. did not significantly diminish the rate of liberation of sialic acid from ganglioside as compared to that of neuraminidase acting on ganglioside in the absence of toxin. The concentration of toxin was sufficient to combine with all the ganglioside present; the concentration of neuraminidase was *c.* 0·01 μg./ml. We were unable to test the reverse concentrations of toxin and neuraminidase because of the limited supply of pure neuraminidase at our disposal.

DISCUSSION

Differences in the ability of various gangliosides to inhibit viral haemagglutination appear to reside in differences in sialic acid content rather than in the presence or absence of amino acid residues as suggested by Rosenberg *et al.* (1956). Not all of the sialic acid which is responsible for viral inhibition is necessarily neuraminidase-sensitive, for comparison of the G0·5 and 'slow' preparations shows the proportion of neuraminidase-sensitive sialic acid to be much lower in G0·5 than in 'slow', while the latter, if anything, had slightly less viral inhibitory capacity.

The gangliosides having a greater sialic content are 'slow' and metachromatic on paper chromatography, and Figs. 1 and 3 (as well as Fig. 6 in Van Heyningen & Miller, 1961) show that there are apparently two such gangliosides. There is a suggestion in Fig. 3 that the second of these gangliosides is the same as G0·5. Since the G0·5 has 33 % neuraminidase-sensitive sialic acid while the mixture of the first and second components (i.e. 'slow') has 57 % neuraminidase-sensitive sialic acid, the proportion of neuraminidase-sensitive sialic acid in the first component is probably greater than 57 %. It is unlikely that the two metachromatic gangliosides are identical with the two gangliosides of Klenk & Gielen (1960) since these latter are 'fast' and non-metachromatic (Van Heyningen & Miller, 1961).

The work reported in this paper confirms the observation of Van Heyningen & Miller (1961) that tetanus toxin-fixation by ganglioside is dependent upon sialic acid content. Although not all the sialic acid of ganglioside is concerned in viral inhibition, it does all appear to be concerned in toxin-fixation. Thus, for example, G2·0, which has practically no viral inhibitory capacity and no neuraminidase-sensitive sialic acid, nevertheless fixes tetanus toxin in proportion to its sialic acid content. Similarly, when 'slow' ganglioside is treated with neuraminidase it retains its toxin fixing capacity in proportion to its residual sialic acid.

It is notable that there are sialic acid-containing substances having biological properties that are the reverse of those of the G2 type of ganglioside, for example, ovomucoid which is an effective inhibitor of viral haemagglutination but does not fix tetanus toxin (Van Heyningen & Miller, 1961).

The figures for the neuraminidase-sensitive sialic acid are subject to some variation and may not in every case represent maximal values, for the tendency of ganglioside to form aggregates may render them inaccessible to the enzyme bonds which would otherwise be attacked. There seem to be at least two possible explanations for differences in neuraminidase sensitivity of sialic acid in ganglioside: there may be more than one kind of sialic acid linkage in ganglioside, or, the gangliosides themselves may form different types of micelles in which the availability of the bonds to the enzyme differs.

In addition to the acknowledgements made in the preceding paper by van Heyningen & Miller (1961) we wish to thank Professor R. Kuhn for samples of gangliosides, Dr G. L. Ada for crystalline neuraminidase, and Dr A. Isaacs for influenza virus and helpful advice. This work was in part supported by a grant (to W. E. van H.) from the Office of Naval Research of the United States Department of the Navy (Project No. 103-474). Grateful acknowledgement is made to the Commonwealth Fund for a Fellowship granted to A. W. B.

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Further Studies on the Repression of Methionine Synthesis in *Escherichia coli*

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SUMMARY

The observation that growth in the presence of methionine results in organisms with a greatly decreased ability to form methionine from homocysteine has been extended to other strains of *Escherichia coli*, in particular strain PA15, an organism for which there is a considerable background of knowledge concerning the enzymic mechanism of this stage of methionine synthesis. When organisms grown in the absence of methionine ('active' organisms) are transferred to a medium containing methionine the activity is not lost, but simply diluted by new organisms having much less activity. Both organisms and ultrasonic extracts derived from them show the same comparative loss of activity when the organisms are incubated with methionine.

Organisms grown in the presence of methionine ('inactive' organisms) regain activity when incubated in a growth medium without methionine. The major part of such regeneration of activity occurs before growth is significant. Enzyme formation occurs linearly and without lag and is stopped by the addition of methionine at any stage. A number of analogues of methionine also repress enzyme formation, but none is as active as methionine.

Regeneration of enzyme is inhibited by chloramphenicol and is greatly diminished when the supply of individual amino acids is restricted either by the use of an inhibitory analogue (*p*-fluorophenylalanine) or by the use of amino acid-requiring auxotrophs. Formation of enzyme therefore requires *de novo* synthesis of protein. Similar techniques gave no evidence that concurrent synthesis of ribonucleic acid was also required.

Compounds which act as substrates or cofactor for methionine synthesis (serine, homocysteine, cobalamin) also repressed enzyme formation to some extent and, after a lag, full repression was obtained with a mixture of all three. These effects are probably due to methionine itself whose synthesis by the enzyme initially present is stimulated by the test substances.

INTRODUCTION

In earlier work in this laboratory it was shown that growth of a number of strains of *Escherichia coli* in a medium containing methionine resulted in an almost total loss by the organisms of their ability to form methionine from homocysteine and serine. Furthermore, strong evidence was obtained that the change was a purely phenotypic one (Wijesundera & Woods, 1953, 1960). The phenomenon thus appeared to be a typical case of enzyme repression (Vogel, 1957) by the ultimate product of the activity of the enzyme in question. All the earlier work, and also that of Cohn, Cohen & Monod (1953), had been carried out with intact organisms and it remained theoretically possible that the failure to metabolize homocysteine after growth on methionine was due to a loss of the ability of homocysteine to permeate the organism. Part of the object of the present work was to determine by experiments with cell-free enzyme preparations whether enzymic activity for the conversion of homocysteine to methionine was also lacking or reduced after culture on methionine.

It was also shown (Wijesundera & Woods, 1960) that a single subculture in the absence of methionine completely restored the ability of the organisms to synthesize this amino acid. A second object of the present work was to determine under more controlled conditions the factors necessary for the production of the enzyme, and particularly whether *de novo* synthesis of protein occurred. A similar study of the synthesis of the enzyme ornithine transcarbamylase was made by Maas & Gorini (1957); the formation of this enzyme, which is concerned in the synthesis of arginine, is repressed by growth of the organism (*Escherichia coli*) in the presence of arginine.

Following the terminology of Wijesundera & Woods (1960) 'inactive' organisms will be used in reference to organisms harvested after growth in the presence of methionine and having severely restricted ability to convert homocysteine to methionine; 'active' organisms are those which have not been exposed to methionine.

The strain (PA15) of *Escherichia coli* used for most of the work was chosen because there was a considerable background of knowledge, both with intact organisms and cell-free enzyme preparations, concerning the mechanism of the reaction between homocysteine and serine which yields methionine (Gibson & Woods, 1960; Szulmajster & Woods, 1960; Kisliuk & Woods, 1960; Guest, Helleiner, Cross & Woods, 1960).

METHODS

Organisms. Escherichia coli PA15 is an auxotroph requiring serine or glycine for growth; it was obtained originally from Dr Barbara Wright. Other strains used for certain experiments required for growth arginine and uracil (C21), thymine (15T$^-$) and homocysteine or methionine (26/18). All strains were maintained on tryptic digest of meat agar slopes, subcultured monthly and stored at 4° after incubation for 18 hr. at 37°.

Preparation of suspensions of organisms. The basal medium (GL) used throughout was the glucose + lactate medium of Guest *et al.* (1960); it was normally supplemented with glycine (10mM) for strain PA15, though DL-serine (10 mM) was used in some experiments. Other supplements were: uracil (mM) + L-arginine (10 mM) for strain C21; thymine (mM) for strain 15T$^-$; DL-homocysteine (mM) for strain 26/18).

The inoculum for the main cultures was developed as follows. Medium GL (6 ml.) containing the appropriate growth factor was dispensed in 150 × 150 × 16 mm. ⊥-shaped tubes (Monod, Cohen-Bazire & Cohen, 1951) and autoclaved at 115° for 7 min. After seeding from an 18 hr. culture on tryptic digest of meat agar the tubes were rocked for 8 hr. at 37° at 36 oscillations/min. of excursion 10 cm.

The main cultures were grown in 1 l. conical flasks containing 200–500 ml. of medium GL supplemented with the appropriate growth factors and autoclaved at 115° for 7 min. After inoculation the flasks were shaken (circular motion, 220 rotations/min. of radius 2 cm.) in air at 37° for 16–18 hr. The size of the inoculum was so adjusted (0·05–0·5 ml. of the liquid culture described above) that the culture density with the various strains reached the equivalent of 0·2–0·7 mg. dry wt. organism/ml. in the time stated. These precautions were taken in order to obtain young cultures of approximately similar physiological age with all strains. The organisms were harvested by centrifuging (15 min. at 2000 *g*) and washed once with the culture volume of water. For direct tests of ability to synthesize methionine they were resuspended at a concentration equiv. 0·3–0·4 mg. dry wt./ml. in 0·066 M-potassium phosphate buffer pH 7·4.

Preparation of extracts of organisms. Enzyme-containing extracts were prepared by resuspending washed organisms in water (20–30 mg. dry wt./ml.) and subjecting to ultrasonic vibration and further treatment as described by Guest *et al.* (1960). The protein content of the final preparation was determined spectrophotometrically according to Layne (1957). Extract of heated *Escherichia coli* (strain PA15) was also prepared according to Guest *et al.* (1960).

Study of enzyme formation. Organisms were harvested from medium GL (supplemented with appropriate growth factors) but containing also 10 mM-DL-methionine. After washing as above they were resuspended at about 0·3 mg. dry wt./ml. in fresh medium, but with methionine omitted. The suspension (100 ml. in 1 l. conical flasks) was shaken at 37° as described for the growth of cultures. Samples were removed at intervals (usually hourly) to determine the extent of growth and the ability to synthesize methionine. In the latter case the organisms were centrifuged out, washed with the sample volume of water, resuspended in 0·066 M-potassium phosphate buffer (pH 7·4) and assayed as described below. In some experiments cell-free enzyme activity was also measured: in this case it was necessary to have larger samples and to start with a larger (250 ml.) volume of suspension. Ultrasonic treatment was as described above.

Measurement of the synthesis of methionine. In the case of intact organisms the procedure was similar to that of Gibson & Woods (1960). The washed organisms were suspended at a concentration equiv. *c.* 0·17 mg. dry wt./ml. in solution MSA which contained: DL-homocysteine (6 mM) DL-serine (10 mM) (or, in some experiments, glycine (10 mM)), glucose (20 mM), cobalamin (18·4 μmM) and potassium phosphate buffer (pH 7·4; 33 mM). Reactions were carried out in a final volume of 2 ml. After incubation for 3 hr. in air at 37° the suspensions were heated at 100° for 10 min., centrifuged and the supernatant fluids assayed for methionine. A control in which the complete reaction mixture had been heated initially at 100° for 5 min. was included in each experiment.

The test for methionine synthesis by ultrasonic extracts of organisms was based on the procedure of Guest *et al.* (1960). The enzyme at a final concentration of 2·5–5 mg. protein/ml. was added to solution MSB which contained in potassium phosphate buffer (0·125 M; pH 7·8): L-serine (5 mM), DL-homocysteine (5 mM), glucose (10 mM), $MgCl_2$ (5 mM), adenosine triphosphate (5 mM), diphosphopyridine nucleotide (0·5 mM), pyridoxal phosphate (0·25 mM) and either extract of heated *Escherichia coli* (0·1 ml./ml.) or tetrahydropteroylglutamic acid (0·5 mM) + cobalamin

(0·37 μM). In some experiments serine was replaced as donor of the one carbon atom unit by glycine (5 mM) or by the formaldehyde derivative of tetrahydropteroylglutamic acid (1·65 mM); in the latter case formaldehyde was mixed with tetrahydropteroylglutamic acid (Kisliuk & Woods, 1960) before adding to the reaction mixture and the concentration of homocysteine was decreased to a half. The tests were carried out in a final volume of 2 ml. and incubation was at 37° for 3 hr. under an atmosphere of H_2. After heating (100°, 3 min.) the reaction mixture was centrifuged to remove precipitated protein and the supernatant fluid used for the assay of methionine. In each experiment there was a control in which the complete reaction mixture was heated initially to 100° for 5 min.

Methionine was determined microbiologically with *Leuconostoc mesenteroides* (*Streptococcus equinus*) P60 as described by Gibson & Woods (1960). It was found incidentally that this organism responds as well to DL-methionine sulphoxide as to DL-methionine.

Measurement of mass of organisms. Extent of growth and the dry weight of organisms was assessed as described by Wijesundera & Woods (1960) using an EEL photoelectric colorimeter (Evans Electroselenium, Halstead, Essex). For strain PA15 a reading of 15 corresponded to a dry weight of 0·5 mg./ml.

Expression of results. Since the interest was in enzyme activity per unit of organism rather than in total enzyme present in a culture or suspension, results have been expressed as μmole L-methionine formed/mg. dry wt. organism. In the case of cell-free extracts the basis used is mg. protein of the extract.

Chemicals. Most of the special chemicals used were as described by Guest *et al.* (1960). DL-Methionine sulphoxide was a laboratory specimen (m.p. 237° uncorrected) whose origin could not be traced. The sources of other analogues of methionine were: DL-α-methylmethionine (Sigma Chemical Co., St Louis, Mo., U.S.A.); L-S-methyl-cysteine (California Corporation for Biochemical Research, Los Angeles, U.S.A.); DL-methionine sulphone (Nutritional Biochemicals Inc., Cleveland, Ohio, U.S.A.); DL-ethionine (Roche Products Ltd., Welwyn Garden City, Herts).

The analogues and other amino acids were tested for possible contamination with methionine by assay with *Leuconostoc mesenteroides* P60. They were also tested by chromatography on paper; descending chromatograms were developed on Whatman no. 1 paper with a saturated solution of water in collidine and with ninhydrin (2 % in *n*-butanol saturated with water) as detecting agent. Methionine and possible impurities were used as markers.

RESULTS

Since the strains of *Escherichia coli* used in the present work differed from those studied by Wijesundera & Woods (1960) it was first necessary to establish that growth in the presence of methionine also decreased the ability of the present strains to synthesize methionine from homocysteine. This proved to be the case (Table 1), 90–100 % repression being obtained with strain PA15 and two other strains; the repression was, however, only about 60 % with the strain requiring either homocysteine or methionine for growth.

With the one strain tested (PA15) the ability of organisms grown without methionine to synthesize this amino acid varied somewhat with the age of the culture from which the organisms were harvested. It was maximal at a culture density of 0·5 mg. dry wt./ml., but was only 30 % at 1·5 mg. dry wt./ml. Young cultures were therefore used for the remaining work.

Table 1. *Effect of growth with methionine on the synthesis of methionine by various strains of* Escherichia coli

Organisms grown on medium GL (plus stated growth factor) with and without DL-methionine (10 mM). Organisms were harvested at a similar stage of growth and tested for methionine synthesis in solution MSA; incubated 3 hr.

Strain	Growth factor(s) added	L-Methionine formed (μmole/mg. dry wt.) Methionine absent during growth	 Methionine present during growth	% decrease when methionine present during growth
PA15	Glycine	2·40	0·10	96
C21	Uracil + arginine	0·90	0·07	92
15T$^-$	Thymine	1·05	0·11	90
26/18	Homocysteine	0·84	0·31	63

Table 2. *Effect of growth on methionine on the activity of ultrasonic extracts of* Escherichia coli *PA* 15

Organisms were grown on medium GL + glycine with the stated concentration of methionine. Ultrasonic extracts prepared from the harvested organisms and assayed for methionine synthesis in solution MSB. Cobalamin (0·37 μM) was present in all tests in which tetrahydropteroylglutamate or its formaldehyde derivative was used. Incubated 3 hr. EHC = extract of heated *E. coli*; PtH$_4$G = tetrahydropterolyglutamic acid and PtH$_4$G-CH$_2$O = its formaldehyde derivative.

C$_1$ donor	Serine	Serine	PtH$_4$G -CH$_2$O	Glycine	Glycine
Folic acid cofactor ...	EHC	PtH$_4$G		EHC	PtH$_4$G
DL-Methionine (M) in growth medium	L-Methionine formed (μm mole/mg. protein)				
0	170	180	61	40	40
10^{-6}	158	173	57	39	—
10^{-5}	141	151	51	39	—
10^{-4}	122	117	35	31	—
10^{-3}	34	34	20	18	—
10^{-2}	22	25	17	15	10

Comparable figures (μm mole L-methionine/mg. dry wt.) for whole organisms tested with serine as C$_1$ donor were (M concentration of DL-methionine in growth medium in parenthesis) 1700 (0), 1500 (10^{-6}), 1300 (10^{-5}), 780 (10^{-4}), 240 (10^{-3}) and 140 (10^{-2}).

Enzymic extracts of organisms grown in the presence of methionine

Guest *et al.* (1960) established three conditions for the synthesis of methionine from homocysteine by ultrasonic extracts of *Escherichia coli* PA15 according to the nature of the donor of the C$_1$ unit and of the source of folic acid acting as the cofactor for the C$_1$ transfer reaction. These were: (1) serine as donor and extract of heated *E. coli* as cofactor; (2) serine as donor and tetrahydropteroylglutamate (in the presence of cobalamin) as cofactor; (3) the formaldehyde derivative of tetrahydropteroylglutamate (again in the presence of cobalamin) both as donor and cofactor. Extracts of organisms grown in the presence of a range of concentrations of methionine were tested under all three conditions; in addition glycine was used as C_1 donor in place of serine under conditions (1) and (2). In all cases growth in the presence of methionine at 10 μM and above led to significant decrease in enzyme activity; at 10 mM the decrease reached 80–90 % (Table 2). The sharpest increase in repression occurred between 0·1 mM and mM methionine. The maximum degree of repression was not, however, quite so great as that observed with intact organisms (about 95 %). Mixtures of ultrasonic extracts prepared from 'active' and 'inactive' organisms had an activity equal to the sum of the separate activities; the 'inactive' extracts therefore did not contain an inhibitor.

Synthesis of methionine with glycine as C_1 donor is less active than with serine, but marked repression was again obtained (Table 2). Growth of strain 26/18 on homocysteine instead of methionine increased the activity of ultrasonic extracts by two- to threefold.

Loss of ability to synthesize methionine

'Active' organisms were resuspended in fresh medium GL+methionine and incubated with shaking. Samples were removed, the organisms re-harvested and again tested for their ability to synthesize methionine. The specific activity of the organisms decreased as would be expected if new enzyme were formed only in the restricted amount anticipated for organisms growing with methionine. The total activity present in the culture rose only slowly and the activity per unit organism decreased theoretically with the increasing amount of new growth (Fig. 1). The theoretical curve in Fig. 1 was computed on the assumption (from previous experiments) that the specific activity of organisms grown in the presence of methionine is 0·12 μmole L-methionine/mg. dry wt./3 hr. Then if the original activity of the organisms grown without methionine is a μmole L-methionine/mg. dry wt./3 hr., then the specific activity after n generations is

$$a/2^n + 0{\cdot}12(2^n - 1)/2^n.$$

A control in which the organisms were resuspended in medium GL without methionine showed no significant changes in specific activity (Fig. 1).

When the harvested organisms were re-incubated with methionine in conditions in which no growth occurred (mM-2:4-dinitrophenol present or source of nitrogen omitted from medium GL) the specific activity of the organisms fell only very slowly (15 % in 5 hr., the time equivalent to three generations when growth occurred.

Release from repression; formation of the enzyme by growing cultures

'Inactive' organisms were harvested after growth with methionine (10 mM) and resuspended in fresh medium without methionine at a density about equal to that of the original culture at the time of harvesting. The organisms rapidly regained ability to synthesize methionine from homocysteine (Fig. 2) and even before detectable multiplication began there was usually a six- to tenfold increase in activity. A control batch of organisms resuspended in medium containing methionine maintained its low order of activity, though growth was similar to that in the culture without methionine (Fig. 2). Ultrasonic extracts prepared from samples taken during the period of increasing activity of the whole organisms also showed a large increase in specific activity (Fig. 3).

Effect of methionine. Addition of methionine at any stage during the recovery of enzyme activity caused the rapid cessation of new enzyme formation and the specific activity ultimately decreased as expected for the dilution of existing organisms by new organisms containing only limited enzyme activity (Fig. 4).

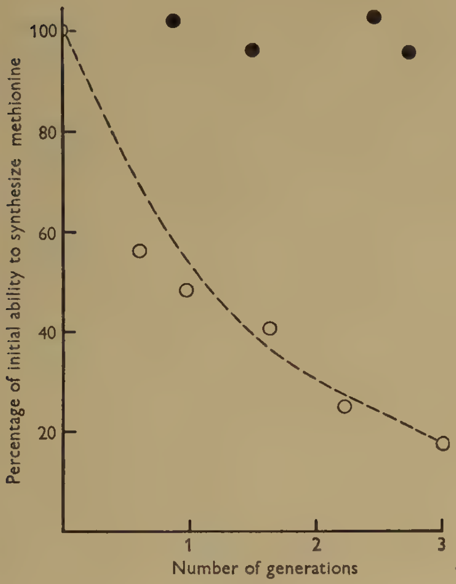

Fig. 1

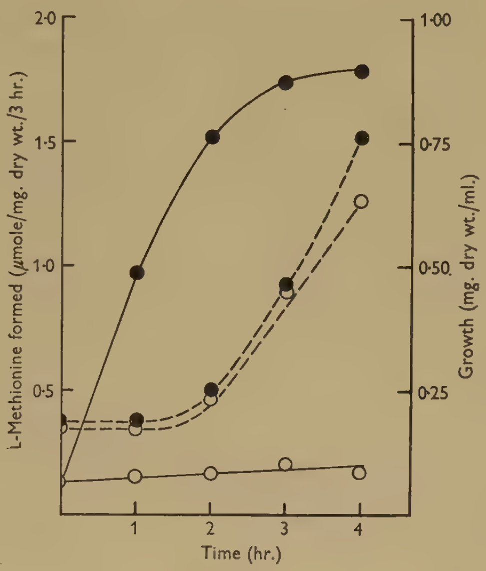

Fig. 2

Fig. 1. Effect of the transfer of organisms grown without methionine to fresh medium with or without methionine. Organisms were harvested from medium GL and resuspended (0·2 mg. dry wt./ml.) in medium GL with (○) or without (●) 10 mM-DL-methionine and incubated with shaking at 37°. Ability of organisms harvested from samples to synthesize methionine was tested in solution MSA. Theoretical curve for ○ (see text) indicated by – – –.

Fig. 2. Effect of the transfer of organisms grown with methionine to fresh medium with or without methionine. Organisms were harvested from medium GL and resuspended (0·2 mg. dry wt./ml.) in medium GL with (○) or without (●) 10 mM-DL-methionine and incubated with shaking at 37°. ———, ability to synthesize methionine (tested in solution MSA). – – –, growth.

The initial decrease was not so great as expected, indicating that there was a lag period before methionine exerted its full effect. This lag was more apparent when methionine was added at 1 hr. (Fig. 4) since new growth was negligible between 1 and 1·5 hr. Graded concentrations of methionine were also added initially before incubation; the extent of recovery of enzyme activity decreased with increasing methionine concentration, the sharpest change occurring between μM and 10 μM (Table 3).

Effect of chloramphenicol. Recovery by 'inactive' organisms of their ability to synthesize methionine when transferred to a medium without methionine was

Table 3. *Effect of the initial addition of methionine on the recovery of enzyme activity*

'Inactive' organisms (strain PA15) harvested from growth in the presence of DL-methionine (10 mM). Resuspended (0·3 mg. dry wt./ml.) in medium GL + glycine and the stated concentrations of DL-methionine. Organisms re-harvested from samples and tested for methionine synthesis in solution MSA.

DL-Methionine added to suspension (M)	L-Methionine formed (μmole/mg. dry wt./3hr.) by organisms after incubation of suspensions for (hr.)		
	0	1	3
0	0·13	0·85	1·85
10^{-6}	0·13	0·7	1·82
10^{-5}	0·13	0·34	0·39
10^{-4}	0·13	0·20	0·28
10^{-3}	0·13	0·18	0·24
10^{-2}	0·13	0·15	0·16

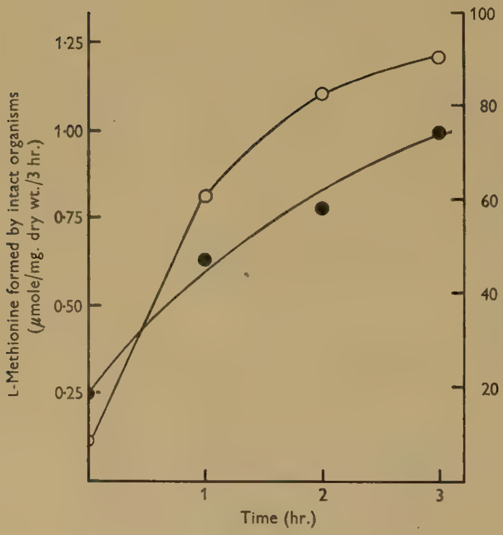

Fig. 3

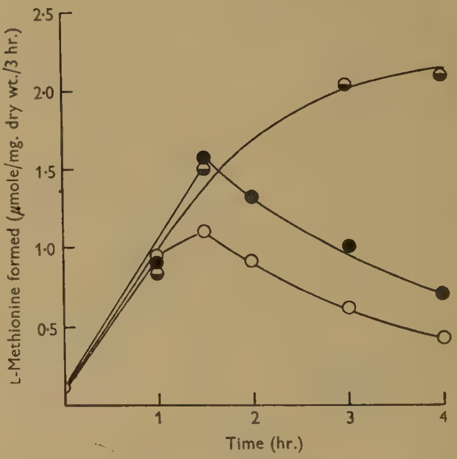

Fig. 4

Fig. 3. Effect of the transfer of organisms grown with methionine to fresh medium without methionine on the activity of ultrasonic extracts. Procedure as for Fig. 2 except that ultrasonic extracts were also prepared from the samples and tested for methionine synthesis in solution MSB. Activities: ○, intact organisms; ●, ultrasonic extract with DL-serine as C_1 donor and extract of heated *Escherichia coli* as cofactor.

Fig. 4. Effect of the addition of methionine at intermediate stages in the recovery of enzyme activity. 'Inactive' organisms (strain PA15) were harvested from growth with DL-methionine (10 mM), resuspended (0·25 mg. dry wt./ml.) in fresh medium without methionine and incubated. DL-methionine (10 mM) was added after 1 hr. (○), 1·5 hr. (●) or not at all (control) (◒).

completely prevented by chloramphenicol; furthermore, the antibiotic also stopped enzyme production when added at intermediate stages of the recovery (Fig. 5). Since chloramphenicol at the concentration used also almost completely inhibited growth, the specific activity of the enzyme in this case remained constant or decreased slowly rather than showing the sharp decline which occurs when new organisms dilute the existing activity. Chloramphenicol is known to inhibit protein synthesis and these results suggest that there is *de novo* synthesis of enzyme protein when the organism recovers its ability to convert homocysteine to methionine.

Effect of amino acids. At the concentration used in most experiments (mM), *p*-fluorophenylalanine inhibited growth of the suspensions completely when added initially. There was very slight growth (10 % of the control) when the addition was delayed for 1 hr. Inhibition of growth was not however material from the present point of view since, as noted above, considerable enzyme formation occurred initially under the experimental conditions without detectable growth. Recovery of activity in synthesizing methionine was decreased by 80 % when *p*-fluorophenylalanine (mM) was added initially, while it was stopped completely when the addition of analogue was delayed for 1 hr., i.e. until recovery was already in progress (Table 4). A lower concentration of *p*-fluorophenylalanine (0·3 mM) inhibited enzyme production by about 60 %. The inhibitory effect of *p*-fluorophenylalanine was overcome completely by an equimolar concentration of phenylalanine, indicating that the inhibition specifically concerned the utilization of the latter amino acid. This is again in accord with the hypothesis that recovery is dependent on *de novo* synthesis of protein.

Table 4. *Effect of* p-*fluorophenylalanine on the recovery of enzyme activity*

'Inactive' organisms (strain PA15) were harvested from growth in the presence of DL-methionine (10 mM), resuspended (0·4 mg. dry wt./ml.) in medium GL + glycine and incubated with the supplements stated (added initially unless otherwise indicated). Organisms were re-harvested from samples and tested for methionine synthesis in solution MSA.

		L-Methionine formed (μmole/mg. dry wt./3 hr.) by organisms after incubation of suspension for (hr.)		
Expt.	Additions to suspension	0	1	3
1	None (control	0·13	0·82	1·99
	p-Fluorophenylalanine (mM)	0·13	0·40	0·50
	p-Fluorophenylalanine (0·3 mM)	0·13	0·56	0·73
	p-Fluorophenylalanine (mM) + phenylalanine (mM)	0·13	0·86	1·98
2	None (control)	0·12	0·42	1·34
	p-Fluorophenylalanine (mM)	0·12	0·29	0·41
	p-Fluorophenylalanine (mM)*	0·12	0·58	0·48

* Added after incubation in progress for 1 hr.

Another method by which the amount of a given amino acid present in a culture may be strictly controlled is by the use of auxotrophic strains which cannot synthesize the amino acid in question. Strains PA15 and C21 required for growth glycine (or serine) and arginine, respectively; 'inactive' organisms of both strains showed greatly diminished recovery of ability to synthesize methionine when the required amino acid was omitted from the suspension (Table 5). In the one case tested (strain PA15) the extent of recovery was proportional to the concentration of amino acid (glycine) added. The fact that limited recovery occurred in the absence of added amino acid may have been due to some being present within the organism at the beginning of the experiment; the organism had, of course, been grown originally on a medium containing the required amino acid.

Table 5. *Effect of the absence of certain amino acids on the recovery of enzyme activity*

'Inactive' organisms were harvested after growth on medium GL + DL-methionine (10 mM) and glycine (strain PA 15) or arginine + uracil (strain C21). Resuspended (0·4 mg. dry wt./ml.) in medium GL containing the supplements stated and, in the case of strain C21 only, uracil (mM). Organisms re-harvested from samples and tested for methionine synthesis in solution MSA.

		L-Methionine formed (μmole/mg. dry wt./3 hr.) by the organisms after incubation of suspension for (hr.)		
Strain	Addition to suspension	0	1	3
PA 15	None	0·13	0·33	0·49
	Glycine (0·05 mM)	0·13	0·71	0·64
	Glycine (0·2 mM)	0·13	0·96	0·93
	Glycine (mM)	0·13	1·20	1·45
C21	None	0·07	0·18	0·13
	Arginine (10 mM)	0·07	0·30	0·42

Table 6. *Effect of the absence of certain pyrimidines on the recovery of enzyme activity*

'Inactive' organisms harvested after growth on medium GL + DL-methionine (10 mM) and thymine (strain 15T⁻) or arginine + uracil (strain C21). Resuspended (0·5 mg. dry wt./ml.) in medium GL containing the supplements stated and, in the case of strain C21 only, L-arginine (10 mM). Organisms re-harvested from samples and tested for methionine synthesis in solution MSA.

		L-Methionine formed (μmole/mg. dry wt./3 hr.) by organisms after incubation of suspensions for (hr.)		
Strain	Additions to suspension	0	1	3
C21	None	0·07	0·25	0·54
	Uracil (mM)	0·07	0·30	0·50
15T⁻	None	0·10	0·49	0·96
	Thymine (mM)	0·10	0·57	1·02

Effect of pyrimidines and purines. Auxotrophic strains requiring uracil and thymine respectively for growth were used to examine whether the presence of pyrimidines was necessary for the recovery of enzyme activity by 'inactive' organisms. Recovery occurred without addition of uracil (Table 6). Thymine was also not necessary and its presence did not significantly affect the rate of formation of new enzyme (Table 6).

Possible requirements of purines and pyrimidines for regeneration of the enzyme were also explored with strain PA 15 by the use of potentially inhibitory analogues of these substances. 8-Azaguanine (2 mM) did not prevent 'inactive' organisms regaining activity under the usual conditions nor did it affect the growth rate of the suspensions under test; it did, however, increase markedly the lag phase in growth tests with small inocula. 8-Azaxanthine (0·2 mM) was also without effect on regeneration of enzyme, as were also three pyrimidine analogues (2-thiouracil, 2-thiocytosine and 2-thiothymine, each tested at 0·2 mM). The last four compounds at the concentration tested also did not significantly affect the growth rate of the suspensions.

Source of energy. 'Inactive' organisms failed to regenerate the enzyme when glucose and lactate were omitted from the medium in which the organisms were suspended or when an uncoupling agent (mM-2:4-dinitrophenol) was added to the medium. There was also no growth under either condition.

Effect of analogues of methionine on the production of the enzyme

These tests were also carried out in the test system in which harvested 'inactive' organisms regain ability to synthesize methionine when transferred to a medium not containing the amino acid. Norvaline and norleucine were included among the compounds tested since they inhibit the growth of strain PA15, such inhibition being overcome specifically by methionine. All the analogues tested were free from methionine as judged by chromatography on paper and (except in the case of methionine sulphoxide which could not be tested in this way) assay with *Leuconostoc mesenteroides* P60.

Table 7. *Effect of analogues of methionine on enzyme formation*

'Inactive' organisms (strain PA15) were harvested from growth on medium GL + DL-methionine (10 mM) + glycine. Resuspended (0·35 mg. dry wt./ml.) in medium GL + glycine and the supplements indicated. Organisms re-harvested after incubation for 3 hr. and tested for methionine synthesis in solution MSA.

Compound added to suspension	L-Methionine formed (μmole/mg. dry wt./3 hr.)	Repression of activity (%)
None	2·20	—
DL-Methionine (mM)	0·22	90
DL-Methionine sulphoxide (mM)	0·32	86
DL-Methionine sulphoxide (0·1 mM)	0·53	76
DL-Methionine sulphoxide (0·01 mM)	1·96	11
DL-Methionine sulphoxide (μM)	2·20	0
DL-Methionine sulphone (mM)	0·95	57
DL-Ethionine (mM)	1·38	37
L-S-Methylcysteine (mM)	1·20	45
DL-α-Methylmethionine (mM)	0·92	58
DL-Norvaline (mM)	1·15	48
DL-Norleucine (mM)	0·66	70

Methionine sulphoxide (mM) repressed enzyme formation almost as much as methionine itself at the same concentration (Table 7), but a titration showed that it had at most only a quarter of the activity of methionine (compare Tables 3 and 7). It is probable that the activity of the sulphoxide is due to its conversion to methionine. Sourkes & Trano (1953) showed that methionine sulphoxide was reduced to methionine by suspensions of *Escherichia coli* in the presence of H_2, and auxotrophs of the same organism which require methionine for growth also respond to the sulphoxide (Lockingen, Humphrey & Wyss, 1958; unpublished observations by the present authors). In qualitative tests by the paper chromatographic technique, both intact organisms and ultrasonic extracts of the present test organism (*E. coli* strain PA15) converted methionine sulphoxide partially to methionine.

Methionine sulphone (mM) caused some repression (60 %); it is unlikely that this compound is converted to methionine since it does not support the growth of auxotrophs of *Escherichia coli* which require methionine. The result with ethionine (about 40 % repression) with strain PA15 conflicts with that obtained by Wijesundera & Woods (1960) who found no activity with another strain (Y44).

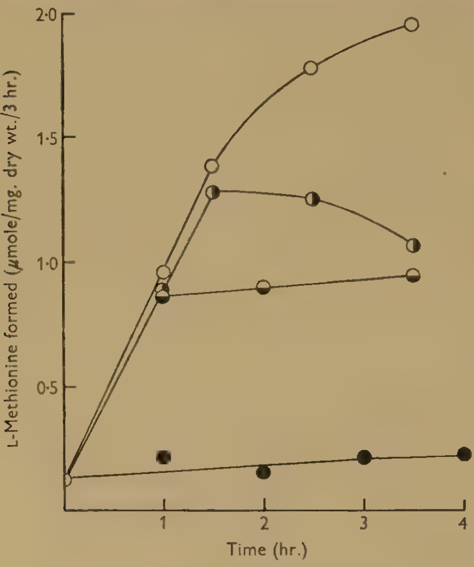

Fig. 5

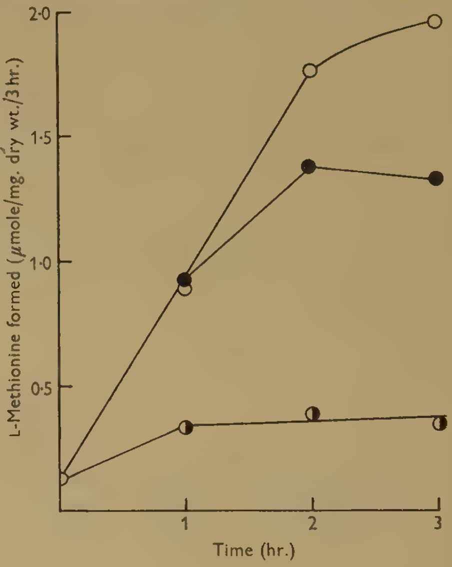

Fig. 6

Fig. 5. Effect of chloramphenicol on the recovery of enzyme activity. Experimental conditions as for Fig. 4. Chloramphenicol (0·125 mM) was added initially (●) or after 1 hr. (◒) or after 1·5 hr. (◑). Control without chloramphenicol (○).

Fig. 6. Effect of serine and other substances required for methionine synthesis on the recovery of enzyme activity. 'Inactive' organisms were harvested from growth on DL-methionine (10 mM) and resuspended in medium GL + the substances indicated. Organisms were re-harvested from samples and tested for methionine synthesis in solution MSA. Substances added to the suspension: 10 mM-glycine (○); 10 mM-DL-serine (●); 10 mM-DL-serine + 5 mM-DL-homocysteine + 15 μmM-cobalamin (◑).

Norvaline and norleucine (both mM) gave 50–70 % repression (Table 7). In growth tests from small inocula both amino acids inhibited growth by about 75 % at 0·1 mM. Under these conditions, however, organisms grown with norvaline had normal ability to convert homocysteine to methionine, while with those grown with norleucine the activity was only about 40 % of that of the controls grown without norleucine.

Effect of substrates for methionine synthesis on the formation of the enzyme

The main substrates required for methionine synthesis (serine and homocysteine) might affect recovery of ability to synthesize methionine in either of two ways. First, they might have intrinsic activity in repressing the formation of the enzyme. Secondly, as the enzyme was formed, their presence might lead to methionine production by the suspension, the methionine in turn repressing the formation of more enzyme; in this case a lag would be expected before the effect became maximal. Cobalamin was included in some of the experiments to be described because it markedly increases synthesis of methionine by suspensions of strain PA15 (Gibson & Woods, 1960).

Strain PA15 will grow when provided with either serine or glycine, though glycine was the supplement used in medium GL for all experiments so far described. The effect on the recovery of enzyme activity of substituting serine for glycine in the medium to which 'inactive' organisms were transferred is shown in Fig. 6. During the first hour of incubation there was no significant difference but later the rate of increase of specific activity decreased in the presence of serine more rapidly than with glycine. When both serine and glycine were present the results were the same as those for serine alone. When the suspension fluid was supplemented with a mixture of serine, homocysteine and cobalamin there was initially only a small increase in enzyme activity and this did not increase further (Fig. 6). The suspending fluid was found to contain 0·05 mM-L-methionine at the end of the experiment. The individual effect of homocysteine was comparable with that of serine though that of cobalamin, though significant, was smaller (Table 8).

Table 8. *Effect of homocysteine and cobalamin on enzyme formation*

'Inactive' organisms (strain PA15) were harvested from growth on medium GL + DL-methionine (10 mM) + glycine. Resuspended (0·3 mg. dry wt./ml.) in medium GL + glycine and the supplements indicated. Organisms were re-harvested from samples and tested for methionine synthesis in solution MSA.

	L-Methionine formed (μmole/mg. dry wt./3 hr.) by organisms after incubation of suspension for (hr.)		
Addition to suspension	0	1·5	4
None	0·10	0·82	1·73
Cobalamin (3 μmM)	0·10	0·78	1·54
DL-Homocysteine (mM)	0·10	0·59	1·28

Similar, though more marked, repression of enzyme activity is found when the test substances are added initially to cultures developed from small inocula. Thus Gibson & Woods (1960) found the activity of organisms (strain PA15) grown on serine to be about half that of organisms grown on glycine. In the present work growth with cobalamin (3 μmM) or DL-homocysteine (mM) gave organisms with only 30 and 25 %, respectively, of the activity of control organisms grown without these substances.

Taken as a whole the results presented in this section suggest that the effects of serine, homocysteine and cobalamin on the development of the enzyme are due to the fact that they cause increased synthesis of methionine which in turn represses the formation of the enzyme system concerned in such synthesis.

DISCUSSION

The major part of the regeneration of enzyme activity takes place before multiplication of organisms has become significant and it occurs without lag and at a constant rate (Fig. 2). It is likely therefore that the new activity appears in the majority of organisms in the population and the conclusion of Wijesundera & Woods (1960) that the change is a phenotypic one is supported. When methionine is added to a suspension of active organisms either initially (Fig. 1) or during the development of activity (Fig. 4) there is no actual loss of enzyme, that present being simply diluted out by new organisms which contain much less enzyme.

Evidence has been obtained (Vogel, 1960) that growth of *Escherichia coli* on arginine represses a system which permits its precursor, acetylornithine, to enter the organism. In the present case repression of the ability of whole organisms to synthesize methionine from homocysteine was accompanied by almost as great a comparative decrease in the activity of cell-free enzyme preparations (Table 2). It is clear therefore that a change to decreased permeability to homocysteine could account at most for a minor part of the present effect.

Limitation of the supply of amino acids, either by the use of auxotrophs unable to synthesize a given amino acid or by the use of an amino acid analogue (*p*-fluorophenylalanine), leads to severe restriction of new enzyme formation. In addition, chloramphenicol inhibits the process either when added initially or during the course of the development of the enzyme. A source of energy is also required. It is likely therefore that *de novo* synthesis of protein from amino acids is required for the regeneration of ability to synthesize methionine rather than it being a case of the reactivation of an inactive or masked protein already present.

There is evidence that the formation by *Staphylococcus aureus* of the inducible enzyme, β-galactosidase, requires the concurrent synthesis of ribonucleic acid (Gale & Folkes, 1955); furthermore, such enzyme formation is inhibited by the purine analogue 8-azaguanine (Creaser, 1956). 8-Azaguanine did not however inhibit β-galactosidase formation in a strain of *Escherichia coli* (unpublished observations of Dr J. Lascelles in this laboratory). Furthermore, ribonucleic acid synthesis does not appear to go on when repressed enzymes are regenerated in *Aerobacter aerogenes* (Magasanik, Magasanik & Neidhart, 1959). In the present case restriction of uracil (by the use of an uracil auxotroph) or of purine (by the use of 8-azaguanine) did not affect regeneration of the ability to form methionine from homocysteine; it is provisionally concluded that concurrent synthesis of ribonucleic acid is not required. Similarly, lack of any effect when thymine was restricted by the use of a thymine-requiring mutant suggested that synthesis of deoxyribonucleic acid was not essential, though its presence is no doubt required.

A number of compounds structurally related to methionine also repressed formation of the enzyme though none approached the activity of methionine itself. The inhibition of growth by norleucine may be partly due to repression of formation of the enzyme which synthesises methionine, since when growth was limited by norleucine the organisms showed 60 % repression of the enzyme. With norvaline, however, higher concentrations were required for repression than for inhibiting growth. Cohen & Jacob (1959) found that repression by methionine was not shown by norleucine-resistant mutants of *Escherichia coli*.

The addition to suspensions capable of regenerating the enzyme of substrates (serine, homocysteine) or a cofactor (cobalamin) for methionine synthesis led to some decrease in enzyme formation. This was more marked when the compounds were added to cultures growing from small inocula and was probably due to stimulation of methionine synthesis by the enzyme initially present followed by repression of new enzyme formation by the methionine when formed. When a mixture of serine, homocysteine and cobalamin was added to a suspension of organisms, enzyme formation ceased almost completely after a short initial lag (Fig. 6) and methionine was detected in the reaction mixture at a concentration known to give repression of enzyme formation. This experiment demonstrates also the practical reality of control of methionine synthesis exercised through enzymic repression.

It is often considered (see, for example, Magasanik, 1959) that enzymic repression would be too slow in action to control the biosynthesis of metabolites and that its physiological function may be rather to enable the organism to attain the most economic enzyme composition for growth in a particular environment. It is also considered that control of metabolite synthesis is probably exercised by the ultimate product through inhibition of enzyme activity (rather than formation) by a feed-back mechanism (see Pardee, 1959). In the case of methionine synthesis no control mechanism of the feed-back type has so far been demonstrated. The concentration of methionine required to achieve marked repression of enzyme synthesis is small (10 μM) and may possibly be reached locally by the organism even when growing in a medium containing no preformed precursors of methionine. It seems possible that in the present case, and during the overall growth of a culture, enzymic repression may well control the amount of methionine formed, as well as adjusting the enzymic constitution of the organism when methionine is already present in the external environment. There is evidence that enzymes are repressed in other biosynthetic pathways, presumably by the endogenously synthesized metabolite, when the organism is growing in a simple medium (Ames & Garry, 1959; Maas & Gorini, 1957).

The present case of enzymic repression differs from many others (see Pardee, 1959) in that methionine inhibits the formation of the enzyme system concerned in the last step in its synthesis rather than one required at an earlier stage. Evidence has recently been obtained (unpublished experiments of R. J. Rowbury) that methionine also represses formation of cystathionase, an enzyme required for the preceding step in methionine synthesis.

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Observations on the Specificities of Extracellular Antigens of the Genera *Aeromonas* and *Serratia*

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SUMMARY

Members of the genus *Aeromonas* produced extracellular toxic antigens which were specific for this group and antisera produced against these antigens could be used to identify these organisms. Strains of *A. liquefaciens*, *A. punctata*, *A. hydrophila* and *A. formicans* were apparently very closely related; differences which existed between them were not greater than differences found among the strains of *A. liquefaciens*. Fresh isolates tended to be strongly haemolytic, produced extracellular toxins which were lethal to mice and elicited haemorrhagic lesions upon injection into the skin of rabbit. Old laboratory cultures tended to be much less haemolytic and less toxigenic. *A. salmonicida* appeared to possess extracellular antigens specific for this species but some strains of *A. liquefaciens* were found to possess this antigen.

Members of the genus *Serratia* were included in this study since Stevenson (1959) suggested that all Aeromonas strains are non-pigmented forms of Serratia. All strains of Serratia examined, regardless of their classification into species, were homogeneous and reacted with antiserum produced with one strain. Moreover, no cross-reaction was found between the antigens of Aeromonas and Serratia and it appeared unlikely that Aeromonas could be regarded as a non-pigmented member of Serratia. No cross-reaction was found between the extracellular antigens of these two genera and the other members of the families Enterobacteriaceae and Pseudomonadaceae and antisera produced with these antigens appeared useful in the identification of Aeromonas and Serratia.

INTRODUCTION

The genus *Aeromonas* is a group of Gram-negative bacilli in the family Pseudomonadaceae; they are usually motile by means of polar flagella. Unlike organisms in the genus *Pseudomonas*, those belonging to the genus *Aeromonas* ferment carbohydrate in a manner similar to that of the coliform bacteria and, therefore, the chief differences between the species of the genus *Aeromonas* and those in the genus *Paracolobactrum* are found in the arrangement of their flagella, in the less active fermentation of carbohydrates by the former, and in their pathogenicity. Although the characteristics of Aeromonas are fairly well established, no definite criterion is available at present to establish species within the genus. Snieszko (1957) used the source of isolation to differentiate three species, *A. liquefaciens*, *A. punctata* and *A. hydrophila*; and *A. salmonicida* is recognized by its pathogenicity to fish, particularly Salmonidae. These criteria are not adequate because similar organisms may be isolated from different sources and the virulence of an organism may be lost after prolonged cultivation on artificial media. Due to the absence of any criterion for establishing a species, several new species have been proposed without adequate comparative study with standard strains of well-established species (Pivnick & Sabina, 1957; Stevenson, 1959).

The present study was initiated to find dependable criteria to establish the species within the genus *Aeromonas*. In the usual bacteriological procedures for identification of a Gram-negative bacillus of the coliform type, biochemical pattern and analyses of cellular antigens by agglutination tests are used. However, a few differences in biochemical reactions are always found even among strains of a well-known species of Aeromonas and it is difficult to determine which reactions should be taken as the differential criteria. The serological tests which use cellular antigens are frustrated by the fact that many strains of the same Aeromonas species possessed different cellular antigens. Finally, the possibility of the production of an extracellular toxin was examined because many Aeromonas strains are known to be pathogenic to cold-blooded animals, and the work of Russell (1898) suggested that the production of a toxin other than the endotoxin was possible. It was found that many fresh isolates of *A. liquefaciens* or *A. hydrophila* produced extracellular toxins, but many old laboratory cultures were relatively non-toxigenic; toxigenicity tests did not identify old laboratory strains. However, even old laboratory cultures produced non-toxic extracellular antigens which were also possessed by fresh isolates and these extracellular antigens appeared to be the most dependable criteria for identification of these Aeromonas strains. The present communication describes these findings.

METHODS

The strains of Aeromonas and Serratia used in this study are listed in Tables 1 and 2, respectively. Most of the strains of Aeromonas were obtained from Dr S. F. Snieszko (U.S. Fish and Wildlife Service, Eastern Fish Disease Laboratory, Leetown, W. Va.) and Dr R. Hugh (George Washington University, Washington, D.C.). Two strains of *A. hydrophila* isolated from sick frogs in this laboratory were also included. The strains of *A. margarita* (Stevenson, 1958) isolated from diseased desert locusts (*Schistocerca gregaria* Fôrsk) and one strain of *Pseudomonas noctuarum* (Lysenko, 1958) were obtained from Dr J. P. Stevenson (Department of Zoology, University College, London). Dr Snieszko stated in a personal communication that he considered that *A. liquefaciens*, *A. punctata* and *A. hydrophila* were physiological variants of one species. *A. margarita* and *P. noctuarum* were considered by Stevenson (1959) to be identical with *S. marcescens*.

All strains of Serratia were obtained from Dr L. S. McClung (Indiana University, Bloomington, Indiana). Many of these strains were from the collection of the late Dr R. S. Breed (New York State Experiment Station, Geneva, N.Y.) and were received after his death. These strains were reclassified by Hamilton (1957) according to his criteria and, as can be seen in Table 2, the designation of the species of *Serratia* differed considerably according to the criteria selected for their classification.

Technique for the production of extracellular antigens. The term 'extracellular antigen' indicates all antigenic substances produced by the organisms which

Table 1. *The list of Aeromonas strains used*

Strain no.	Genus	Species	Obtained from	Original source or source of isolation
U-14	*Aeromonas*	*liquefaciens*	Dr Snieszko*	Guppy
U-15	*Aeromonas*	*liquefaciens*	Dr Snieszko	Guppy
U-21	*Pseudomonas*	*punctata*	Dr Snieszko	Dr Rucker (Seattle)
U-23	*Pseudomonas*	*punctata*	Dr Snieszko	Dr Lassleben (Germany)
U-40	*Aeromonas*	*liquefaciens*	Dr Snieszko	Dr Kluyver
U-41	*Aeromonas*	*liquefaciens*	Dr Snieszko	Dr Kluyver
Canada-1958	*Aeromonas*	*salmonicida*	Dr Snieszko	Salmon
Leetown-1959	*Aeromonas*	*salmonicida*	Dr Snieszko	Salmon
A-H-1	*Aeromonas*	*hydrophila*	This laboratory	Frog
A-H-2	*Aeromonas*	*hydrophila*	This laboratory	Frog
251	*Aeromonas*	*hydrophila*	Dr Hugh†	ATCC 7965 §
513	*Aeromonas*	*formicans*	Dr Hugh	ATCC 13136. Pivnick's strain 258
514	*Aeromonas*	*formicans*	Dr Hugh	ATCC 13137. Crawford's Pacific Grove strain
618	*Aeromonas*	sp.	Dr Hugh	Water of Potomac River
944	*Aeromonas*	*hydrophila*	Dr Hugh	Murray's 6, 1:A-1 from trout
829-5	*Aeromonas*	sp.	Dr Hugh	Water from Rock Creek
832-5	*Aeromonas*	sp.	Dr Hugh	Minced beef
SL 10	*Aeromonas*	*margarita*	Dr Stevenson‡	Desert locust
SL 11	*Aeromonas*	*margarita*	Dr Stevenson	Desert locust
SM 006	*Pseudomonas*	*noctuarum*	Dr Stevenson	Dr Lysenko (Czechoslovakia)

* U.S. Fish and Wildlife Service, Eastern Fish Disease Laboratory, Leetown, W. Va.
† George Washington University, Washington D.C.
‡ Department of Zoology, University College, London
§ American Type Culture Collection.

Table 2. *The list of Serratia strains used*

Strain no. assigned by Hamilton*	Name assigned by Hamilton	Name as received by McClung*	Strain no. as received by McClung	Original source
8	*S. indica*	*S. marcescens*	53A	U. of Wisconsin
11	*S. indica*	*S. marcescens*	53B	U. of Wisconsin
16*a*	*S. marcescens*	*S. rubra*	—	Ohio State Univ.
16*b*	*S. marcescens*	*S. rubra*	—	Ohio State Univ.
59*a*	*S. plymuthica*	*Serratia* sp.	2579	CDC†
60	*S. marinorubra*	*Serratia* sp.	2704	CDC
61	*S. marcescens*	*Serratia* sp.	1155	CDC
62	*S. marinorubra*	*Serratia* sp.	1176	CDC
63	*S. marcescens*	*Serratia* sp.	2268	CDC
113	*S. kiliensis*	*Erythrobacillus* sp.	PI4	Breed collection
139	*S. plymuthica*	*S. plymuthica*	HB	Breed collection
146	*S. plymuthica*	*S. plymuthica*	MW	Breed collection
151	*S. kiliensis*	*S. marcescens*	SMH	Breed collection
230*a*	*S. marcescens*	*Serratia* sp.	SM	Beth Israel Hosp. N.Y.

* Indiana University, Bloomington, Indiana.
† Communicable Disease Center, U.S. Public Health Service.

One strain of *Serratia marcescens* from the stock cultures of this department was included in this study.

could be separated readily from the cells. This antigen was produced with the cellophan plate technique already described (Liu, 1957). It consisted essentially in cutting a sheet of cellophan to fit the bottom of a Petri dish, sterilizing it by steam under pressure and placing it in the dish after the appropriate medium was poured and solidified. After the cellophan sheet was moistened, the wrinkles were carefully stretched out. About two drops of broth culture of the organism were placed on the cellophan and spread over the whole plate with a sterile cotton swab. The plate was incubated aerobically at the optimal temperature of each organism, which was 24° for *Aeromonas salmonicida* and 28° for all other Aeromonas and all Serratia species. The medium used for this purpose was the Trypticase-soy agar (Baltimore Biological Laboratories) enriched with 0·5 % glycerol. At the end of the incubation period, which was usually about 48 hr., 3 ml. sterile saline was used to wash off the growth which was centrifuged for 60 min. at 2000 *g* to remove the bacterial cells. The supernatant fluid containing the extracellular antigen was Seitz-filtered and preserved by the addition of enough thiomersal (Merthiolate, Lilly) to make the final concentration of 1/5000.

Production of antisera to the extracellular antigen. Aeromonas strains U-15, U-40, Canada-1958 and five strains of Serratia representing five species (8, 16*a*, 59*a*, 60 and 113) were used for the production of antisera. Strain U-15 was a relatively recent isolate of *A. liquefaciens* and it produced strong haemolysis on blood agar. U-40 on the contrary was an old laboratory culture which was used by the late Dr A. J. Kluyver as a typical strain of *A. liquefaciens*. It was almost rough in its colonial morphology and the extracellular antigens of this strain were found to be the least toxic among those produced by various strains of *A. liquefaciens*. It was used, therefore, as a representative of *A. liquefaciens* which became rough and non-toxigenic after a prolonged culturing on artificial media. The two strains of *A. salmonicida* did not vary much in their toxigenicity and the strain Canada-1958 was used because it was much more chromogenic and appeared to be more typical of this species.

After sterility tests of the extracellular antigen, it was mixed with an equal amount of Freund's incomplete adjuvant (a mixture of Arlacel and Bayol in the ratio of 1·5/8·5) and injected subcutaneously into rabbits at weekly intervals; 0·2 ml. was the dose for all antigens in the first week and 0·5 ml. in the second week. In the cases of U-40, Canada-1958 and all Serratia strains, 0·5 and 1·0 ml. of antigen without the adjuvant were injected intravenously in the 3rd and 4th weeks. The antigens of U-15 were so toxic that 0·2 ml. injected intravenously killed a rabbit within 8 hr. The preparation was diluted, therefore, ten times for intravenous injection. Beginning in the 3rd week, 0·2, 0·5, 1·0 and 2·0 ml. of the diluted antigen were injected intravenously at 3-day intervals. The sera were obtained 1 week after the last injection and preserved with thiomersal.

Identification tests. The technique used for the identification of these organisms with antisera was essentially that devised by Elek (1948) for the *in vitro* toxigenicity test of *Corynebacterium diphtheriae*. The medium used for this purpose contained Tryptose (Difco) 0·1 %, Casamino acids (Difco) 0·3 %, glycerol 0·5 %, Na_2HPO_4 0·3 %, NaCl 0·2 % and Bacto-agar 1·2 % (pH 7·4). The paper strip contained 0·15 ml. undiluted antiserum. Before inoculation of the organisms the plate was incubated overnight at 37° to ensure a dry surface. The inocula were always taken from fresh cultures on blood agar. The plates which contained *Aeromonas salmonicida* were incubated at 24° because these organisms did not grow well at a higher temperature. All other plates were incubated at 28°.

RESULTS

Intradermal lesions produced by the extracellular antigens of Aeromonas and Serratia

The lesions produced by the extracellular antigens of various species of *Aeromonas* in the skin of rabbit are shown in Pl. 1, fig. 1. The extracellular antigens of U-15, A-H-1 and 514 contained toxins which produced haemorrhagic lesions. This was characteristic of most of the fresh isolates of motile Aeromonas strains, regardless of species. The haemorrhagic lesions appeared within 5 min. of the intracutaneous injection of 0·1 ml. of the crude preparation and were similar to the lesions produced by the toxin of *Pseudomonas pseudomallei* described by Nigg, Heckley & Colling (1955). The lesions reached maximum development within 3 hr. There were a few strains of the motile Aeromonas (such as U-40 and U-21 and mostly old laboratory cultures) which did not produce the haemorrhagic toxin (Pl. 1, fig. 1). It is possible that these strains produced toxin when they were first isolated but had lost this ability after prolonged cultivation on artificial media. The two strains of *A. salmonicida* did not produce the haemorrhagic toxin; since both were relatively recent isolates, the lack of haemorrhagic toxin in their extracellular antigens probably indicates a characteristic of the species.

The lesions produced by the extracellular antigens of Serratia are shown in Pl. 1, fig. 2. Fifteen strains of Serratia representing five species were tested. Preparations from some strains produced only abscesses and redness similar to the lesions produced by the endotoxin of Gram-negative bacteria, but preparations from other strains contained toxin which produced haemorrhagic and necrotic lesions. There was no correlation between pigment production and the production of these toxins, or between the type of lesion produced by a strain and its classification into species.

Neutralization of the toxic effects by antiserum

The haemorrhagic toxin of *Aeromonas hydrophila*, *A. punctata* and *A. formicans* could be neutralized by the antiserum to one strain of *A. liquefaciens* (U-15) as shown in Pl. 1, fig. 3. Quantities of serial two-fold dilutions of antiserum in saline were added to an equal amount of the toxin of *A. hydrophila* and *A. formicans* and the mixtures incubated at 37 for 1 hr.; 0·1 ml. each of the mixtures was injected intracutaneously into the rabbit. It can be seen in Pl. 1, fig. 3, that the toxins of both *A. hydrophila* and *A. formicans* were neutralized by the antiserum of *A. liquefaciens*.

Attempts to demonstrate neutralization by protection tests in mice were not successful since the lethal effect of U-15 toxin was not completely neutralized by its homologous antiserum.

Identification tests with Aeromonas species

In Pl. 2, figs. 4 and 5, are shown some of the results obtained in the agar diffusion identification test. Positive results could be noted readily by the formation near the paper strip of the so-called 'arrow head figure' which was due to precipitation of antigens with their homologous antibodies. Most of the motile strains of Aeromonas used in this study were found to react readily with U-15 antiserum. There were a few strains of *A. liquefaciens* (including U-40) which did not react with U-15 antiserum and instead, they were found to react with Canada-1958 (*A. salmonicida*) antiserum, as shown in Fig. 5.

Identification tests with Serratia species

In their extracellular antigens the members of the genus *Serratia* appeared to be homogeneous. All strains of Serratia used in this study, regardless of their pigmentation and designation into species, gave a reaction with antiserum produced with one strain of Serratia. None of the Aeromonas strains except the two of *Aeromonas margarita* and the strain of *Pseudomonas noctuarum* showed reaction with antiserum of the Serratia group.

Table 3. *Summary of the reactions of various strains of Aeromonas and Serratia to three antisera*

Organisms	No. strains used	No. strains reacting to antisera		
		A. liquefaciens (U-15)	*A. salmonicida* (Canada-1958)	*S. marcescens* (16*a*)
Aeromonas liquefaciens	4	3	1	0
A. hydrophila	4	4	0	0
A. punctata	2	1	1	0
A. formicans	2	2	0	0
A. salmonicida	2	0	2	0
Aeromonas sp.	3	2	1	0
A. margarita	2	0	0	2
Pseudomonas noctuarum	1	0	0	1
S. marcescens	6	0	0	6
S. indica	2	0	0	2
S. plymuthica	3	0	0	3
S. kiliensis	2	0	0	2
S. marinorubra	2	0	0	2

The results of these identification tests are summarized in Table 3. It should be noted that most of the motile strains of Aeromonas belonging to the species *A. hydrophila*, *A. punctata* and *A. formicans* reacted readily with antiserum of *A. liquefaciens* and a few of them reacted with antiserum of *A. salmonicida*. The two strains of *A. margarita* and one strain of *Pseudomonas noctuarum* reacted readily with antiserum of *Serratia marcescens* and therefore these organisms appeared to be merely non-pigmented forms of Serratia, as suggested by Stevenson (1959). However, the generalization that all Aeromonas strains are non-pigmented forms of Serratia could not be substantiated because none of the other strains of Aeromonas showed any cross-reaction with antisera of the Serratia group.

No cross-reaction was observed between *Aeromonas*, *Serratia* and other genera of the families Enterobacteriaceae and Pseudomonadaceae; therefore antisera prepared with extracellular antigens of strains of Aeromonas and Serratia appeared to be useful in the identification of these genera.

DISCUSSION

The evidence presented in this paper indicates that the organisms belonging to the genera *Aeromonas* and *Serratia* produce extracellular antigens specific for each genus and that antisera produced with these antigens can be used to identify organisms belonging to these genera. Members of the genus *Aeromonas* are known to be pathogenic to various cold-blooded animals, such as frogs and fish (Emerson & Norris, 1905), and infection in man has been reported (Caselitz & Buck, 1958). Members of the genus *Serratia* are not generally considered to be animal pathogens but infections with members of this genus have been reported (Wheat, Zuckerman & Rantz, 1951; Vernon & Hepler, 1954). The production of toxic extracellular antigens by these organisms may play some role in the pathogenesis of these organisms.

Strains of Aeromonas which have been determined as *A. liquefaciens*, *A. hydrophila*, *A. punctata* and *A. formicans* are apparently very closely related organisms separated into different species on the basis of the source of isolation and a few biochemical reactions. The differences between these species were not greater than the differences found between the various strains of *A. liquefaciens*. The fresh isolates of this group tended to be strongly haemolytic, produced extracellular toxins and various extracellular enzymes, and most of their extracellular antigens reacted with antiserum against toxigenic strains of *A. liquefaciens*, such as U-15. *A. salmonicida* is a different organism as can be seen by its lower optimal temperature of growth, production of pigment and non-motility. However, the cross-reactions between this species and some strains of motile Aeromonas indicate that all strains of Aeromonas are closely related, and the absence of cross-reaction with other Gram-negative bacilli indicates that organisms belonging to the genus *Aeromonas* are a distinct group.

Members of the genus *Serratia* were included in this study at a later stage because Stevenson (1959) suggested that Aeromonas species are non-pigmented members of the genus *Serratia*. Complete absence of cross-reaction between strains of these two genera would seem to exclude the possibility that Aeromonas can be regarded as non-pigmented forms of Serratia. Stevenson (1959) drew his conclusion because of the similarity of *A. margarita* and *S. marcescens* and did not use typical strains of Aeromonas for comparative study. Since both strains of the so-called *A. margarita* used in this study reacted readily with antisera of the Serratia group and not with those of the Aeromonas group it was obvious that these two strains were non-pigmented members of Serratia and not Aeromonas. The results of the present study indicated that members of the genus *Serratia* were quite closely related in spite of the differences in pigment production and a few biochemical reactions which were used to differentiate them into species. Antisera produced against extracellular antigens of one strain of Serratia will identify practically all strains of Serratia regardless of their classification into species, which, according to Breed (1957), depends on minor differences such as solubility of the pigments and pellicle formation, characters that are variable. Inspection of Table 2 will show how different and confusing the classification of this group can be, depending on the criteria preferred by different workers. In view of the cross-reactions between the antigens of various strains of Serratia, classified into different species, it is doubtful whether any good can be achieved by such a classification. A comparison between the classification of *Corynebacterium diphtheriae* and that of the Serratia species will serve to elucidate this point. The species *C. diphtheriae* has been partially established on the basis of its production of an exotoxin. The species has a certain biochemical pattern by which organisms belonging to it can be identified. Strains of the species ferment glucose, maltose and dextrin but not sucrose or lactose. The species *C. diphtheriae* is further divided into gravis, mitis and intermedius varieties by their colonial morphology and a few other biological characteristics. For example, the gravis variety tends to form a pellicle on the surface of broth and to ferment starch. Neither mitis nor intermedius varieties ferment starch and they do not usually form a pellicle. Mitis is usually haemolytic but intermedius is not. In the case of Serratia the characteristic of pellicle formation was used by Breed (1957) to separate *S. indica* from *S. marcescens*. If this criterion were to be taken for the classification of corynebacteria, a pellicle-forming strain of *C. diphtheriae* of the gravis variety would be assigned the rank of an independent species. Since some gravis strains do not form a pellicle but do ferment starch, another species would have to be created for this kind of strain. Solubility of pigments was used by Breed (1957) as a criterion in the separation of Serratia species, but pigment production may be lost by some strains. The so-called *Aeromonas margarita* is an excellent example of a non-pigmented form which is difficult to identify if pigment production is used as a distinguishing criterion.

The differences that existed between the various strains of Serratia tested, although classified into different species, did not appear to be greater than the differences that can be found among strains of other bacteria placed in a single species. The group as a whole could be placed in a single species and different subspecies might be recognized by the characteristics, such as pigment formation and the production of gas or acetylmethylcarbinol, which are not common to all strains.

The extracellular antigens produced by organisms belonging to the family Pseudomonadaceae have received very little, if any, attention in the past. This is due undoubtedly to the fact that the procedures and techniques used at present for the identification of Gram-negative bacilli are those developed primarily for the identification and classification of members of the family Enterobacteriaceae. The enteric bacteria are not active producers of extracellular enzymes or toxins and, therefore, the classification and identification of these organisms depend primarily on their biochemical reactions and cellular antigens. The members of the family Pseudomonadaceae and the genus *Serratia* (although it is included by Breed & Murray (1957) in the family Enterobacteriaceae) are much more active producers of extracellular antigens and some of these antigens, in being heat labile, have some characters of exotoxins. Both from the viewpoint of identification of the organisms and the understanding of their role in pathogenesis, these antigens deserve more attention than they have been accorded hitherto.

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EXPLANATION OF PLATES

Plate 1

All photographs in these three figures were taken 18 hr. after the injections.

Fig. 1. The lesions produced by the extracellular antigens of various species of Aeromonas in the skin of rabbit. They are (from left to right, upper row); 514 (Crawford's Pacific Grove strain of *A. formicans*), A-H-1 (*A. hydrophila*), U-15 (*A. liquefaciens*), (left to right, lower row); Canada-1958 (*A. salmonicida*), U-21 (*A. punctata*) and U-40 (*A. liquefaciens*).

Fig. 2. The lesions produced by the extracellular antigens of fifteen strains of Serratia representing five species. The antigens of some strains contained haemorrhagic and necrotic toxins while those of the other strains did not. There was no correlation between pigment production, species designation of a strain and its toxin production.

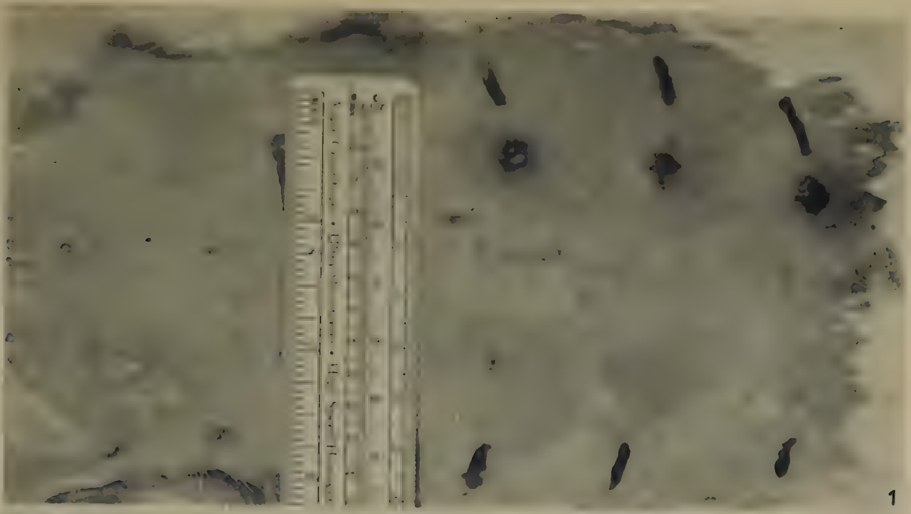
Fig. 3. Neutralization of the haemorrhagic toxins of *A. formicans* and *A. hydrophila* by *A. liquefaciens* antiserum. The upper row are the toxin of 514 (*A. formicans*) added to an equal amount of twofold dilutions of an antiserum of U-15 (*A. liquefaciens*), starting from ½ from left side. 0·1 ml. of the mixtures was injected intracutaneously. The lower row are the toxin of A-H-1 (*A. hydrophila*) added to the same dilution of the same antiserum. The corresponding dilutions of the antiserum are connected by a black line. The control toxins of both species are connected by double lines.

Plate 2

Fig. 4. Five strains of Aeromonas tested with antiserum of U-15 (*A. liquefaciens*). They are (left side of the paper strip from top to bottom): U-15, U-21 (*A. punctata*), U-40 (*A. liquefaciens*); (right side of paper strip from top to bottom), Canada-1958 and Leetown-1959 (both *A. salmonicida*). Note that only the strain U-15 is reacting to this serum (the so-called arrow head figure).

Fig. 5. The same five strains in the same arrangement as shown in Fig. 4 tested with an antiserum of Canada-1958. Note that both strains of *A. salmonicida* are showing strong reactions. The two strains of motile *Aeromonas* (U-21 and U-40) are also showing some reactions, although they are not as strong as those of *A. salmonicida*.

The two photographs in Figs. 4 and 5 were taken after 5 days incubation at 24°.



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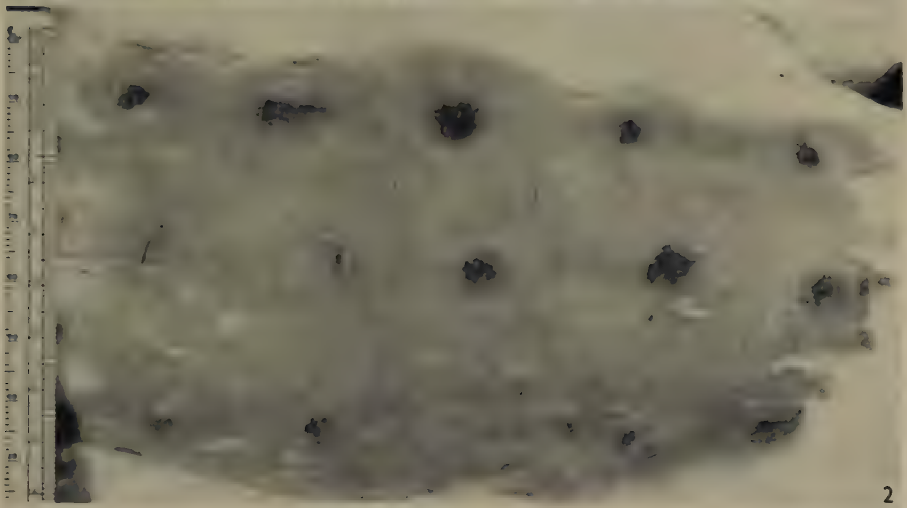

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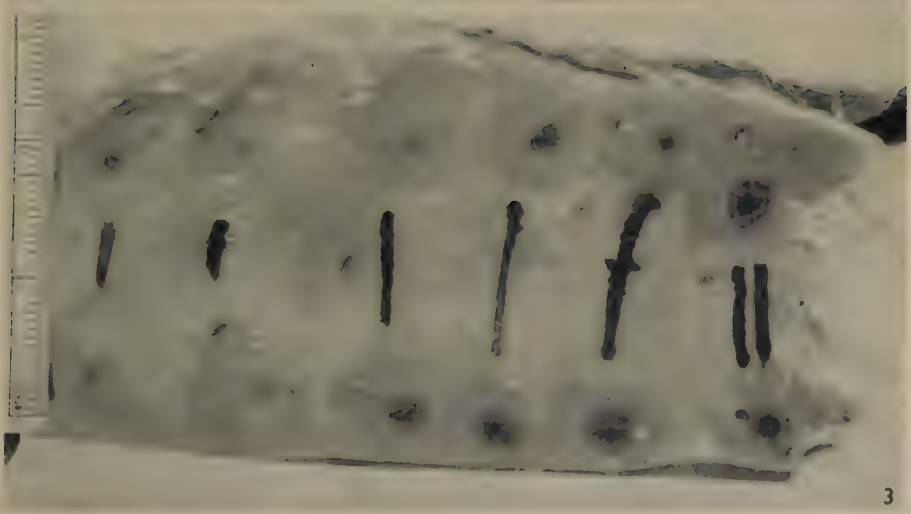

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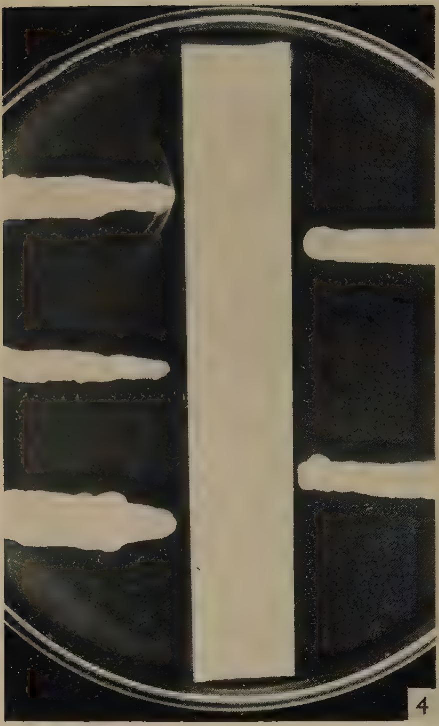

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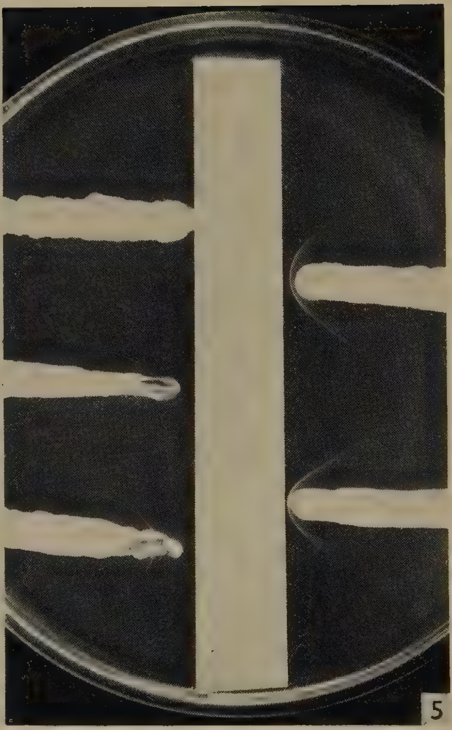

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A Volatile Substance Controlling Early Stages of Zygospore Formation in *Rhizopus sexualis*

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SUMMARY

The inhibitory effect of low temperature (*c.* 10°) on initiation and early development of zygospores of *Rhizopus sexualis* is less severe in mature cultures (in which numerous zygospores have already been formed before transfer to the low temperature) than in young ones. The severe effect of low temperature on immature cultures is not counteracted by any of a number of known growth substances, by extracts of mature mycelium and zygospores, or by used culture media. The effect of mature cultures in counteracting low temperature inhibition of zygospore production in young ones is able to pass across a gap of 5 mm. between the cultures and must therefore be due to a volatile substance (or substances) produced by the mature mycelium. By the use of small chambers which allow young test cultures to be kept at 10° while a stream of air from mature ones growing at 20° passes over them, it is confirmed that the active factor is volatile. This substance is not carbon dioxide or ammonia and is basic in nature. Its probable identity is discussed and comparison is made with some other volatile substances reported to influence growth and development of fungi.

INTRODUCTION

The initiation and early development of zygospores of *Rhizopus sexualis* (Smith) Callen and of some other members of the Mucorales are prevented by temperatures of 10° or lower (Hawker, Hepden & Perkins, 1957). Zygospores which have reached a later stage of development before being subjected to the low temperature continue to develop and eventually reach maturity. Growth of mycelium and sporangia also continues slowly at these low temperatures. Hawker *et al.* (1957) concluded that the inhibition of the early development of zygospores is due to 'a temperature-induced block in the synthesis of some substance or substances essential to further development, or to a slowing up of translocation of such substances into the gametangia from the supporting hyphae'. Occasionally young spores continue to develop in a colony exposed to the critical low temperature when older zygospores are also present. This effect might be due to the presence in relatively old cultures of a reserve of the hypothetical stimulatory substance, the synthesis of which is inhibited by low temperature.

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METHODS

The experimental methods were generally similar to those described in the previous paper (Hawker *et al.* 1957). Special methods will be described in the appropriate places in the text.

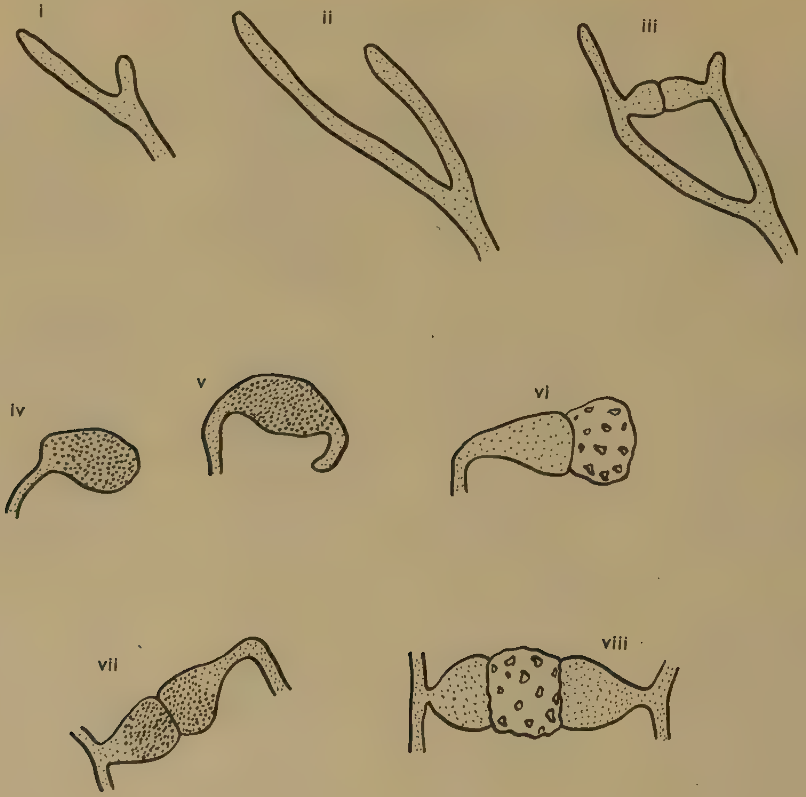

Fig. 1. Normal and abnormal development of zygospores of *R. sexualis*. i, ii, stages in formation of zygophores; iii, production of normal conjugating progametangia from zygophores; iv and v, pseudophores (or abnormal progametangia); vi, azygospore developing from a pseudophore; vii and viii, further development of normal zygospore (for comparison with pseudophore and azygospore). Diagrammatic.

In the earlier paper intensity of reproduction was expressed as the zygospore index (ZI) which was calculated from the numbers of zygospores at various defined stages of development in arbitrarily chosen microscopic fields. In the present investigation the differences were small and the zygospores were not uniformly distributed over the colony so that the use of the zygospore index was not always appropriate. Alternatively, results were expressed as total numbers of zygospores or average number in particular microscopic fields. In many experiments, however, zygospores were not formed, but the zygospores developed swellings (termed pseudophores by Callen (1940)) which have been interpreted as potential progametangia (Fig. 1) or which occasionally develop into azygospores. No method was devised to measure the frequency of these.

The culture used in early experiments was that used throughout the work described in the previous paper. During the present investigations, however, this isolate became variable and showed a general tendency to be less fertile and to produce an increased proportion of azygospores. Through the kindness of Dr Kiyosi Kominami of Tokyo, a new isolate was obtained which was used in all later experiments. The behaviour of this Japanese isolate was similar to that of the original one before the latter deteriorated.

RESULTS

Effect of natural extracts and known growth-promoting substances

Used media and extracts of mycelium. Culture media in which *Rhizopus sexualis* or other fungi had been grown were strained to remove mycelial debris, sterilized by autoclaving or by filtration and solidified with agar. When these used media were re-inoculated with *R. sexualis* growth was sometimes poor and in no example was there any counteraction of the inhibitory effect of low temperature.

Zygospore-bearing mycelium of *Rhizopus sexualis* and of some other members of the Mucorales was homogenized in a small volume of sterile distilled water. Extracts of the homogenate or of the used culture medium were concentrated (i) by evaporation under reduced pressure at 25°, (ii) by rapid evacuation and storage *in vacuo* at low temperature, or (iii) by freezing and removal of ice crystals. These extracts were added to malt agar plates, either by incorporation in the medium or by placing known volumes in holes cut from the agar after the plates had been poured. Again no counteraction of the inhibitory effect occurred.

Extracts of other natural materials. Rhizopus sexualis was grown on extracts of malt (from 1·0 to 3·0 %), potatoes, carrots, raisins, prunes and yeast, either alone or supplemented with nutrient solutions, in liquid form or in media solidified with agar. Only on carrot extract agar was the inhibition at low temperature modified; a few new zygospores were initiated on this medium after transfer to low temperature. In a typical experiment the zygospore index (calculated from twelve arbitrarily chosen microscopic fields in each of three plates) was 46 on 1 % malt extract agar and 157 on carrot agar after 50 hr. at 20°. After a further 72 hr. at 7° these figures rose to 48 and 200, an increase of approximately 4 and 22 %, respectively.

Since carrot contains a number of vitamins it was possible that one or more of these was responsible for the stimulation.

Pure vitamins. A number of vitamins (thiamine, riboflavin, nicotinic acid, pyridoxin, pantothenic acid, cobalamin (B_{12}) and biotin), which are known to influence growth and sporulation of certain micro-organisms, were added to cooling malt extract agar or glucose asparagine agar (glucose, 10 g.; asparagine, 2 g.; KH_2PO_4 1·75 g.; $MgSO_4$, 0·175 g.; distilled water, 1 l.). In other experiments known volumes of solutions of the vitamins were placed in holes cut in plates of agar media in advance of the margin of a growing colony of *Rhizopus sexualis*. None of the vitamins tested, with the exception of B_{12}, decreased the inhibitory effects of low temperature. When a number of zygospores were already present prior to chilling, zygospore initiation continued for a limited time at low temperature when B_{12} was supplied, i.e. the effect of the presence of mature zygospores was enhanced, but not replaced, by this vitamin.

Purines. Robbins & Kavanagh (1942) showed that, for the production of mature zygospores, *Phycomyces blakesleeanus* requires an external supply of hypoxanthine in addition to the thiamin needed for mycelial growth. This result was confirmed. Accordingly the effects of purines on *Rhizopus sexualis* were investigated. In preliminary experiments (Hawker, 1957) there was some evidence that adenine, hypoxanthine and guanine, when added to malt agar or to glucose asparagine agar, increased the rate of growth and of zygospore formation in *R. sexualis* at 20°. Furthermore, these purines showed a partial counteraction of the inhibitory effect of low temperature. In later experiments no such effect could be consistently demonstrated. The experiments were repeated many times by a variety of methods and with a wide range of concentrations of the purines, but the results with *R. sexualis* were usually negative. In a few experiments, in which the source of nitrogen was an inorganic salt, some stimulatory effect of low concentrations of purines occurred. This might be attributed to the use of these as a source of organic nitrogen and not necessarily to a specific stimulatory effect on zygospore initiation.

The effect of the presence of mature living mycelia and zygospores

The stimulatory effect on young colonies of old ones in close contact with them. Since used culture media, mycelial extracts and known growth-promoting substances did not replace mature living zygospores as a means of counteracting the low temperature effect, the influence of old cultures in close contact with young ones was studied. Segments were cut from agar plate cultures in advance of a young colony and were replaced by segments from older colonies of the same or different species. The plates were then placed at 7–10°. Zygospores continued to develop in such colonies. In a typical experiment the numbers of mature zygospores per microscopic field were 6·7 in the sector nearest the inserted segment of the mature colony and 2·6 in the rest of the young colony. No zygospores developed in comparable young colonies in the absence of the mature segment.

In other experiments an old colony of *Rhizopus sexualis*, or of a zygospore-producing species of the homothallic genus *Zygorhynchus*, growing on malt agar, was covered with boiled cellophane over which cool fresh medium was poured. The fresh medium was then inoculated with *R. sexualis*. This method also resulted in the initiation and development of a few zygospores at temperatures below that permitting spore formation in young colonies in the absence of older ones.

These results are consistent with the supply by the mature cultures of small amounts of an essential metabolite, the synthesis of which is prevented by low temperatures. Since this hypothetical substance was lacking in used culture media and mycelial extracts, it must be unstable, or volatile, or both. Attempts were made to detect the presence of such volatile substances.

The stimulatory effect on young colonies of old ones not in contact with them. The segment transfer experiments were repeated but with gaps left between the agar media on which the young (test) colony and the old (source) colony were growing. When this gap was not more than 5 mm. there was some counteraction of the effect of low temperature on zygospore initiation in the young test culture. Since the stimulus could pass over a gap of 5 mm. it is likely to be due to a volatile substance formed by the old colony.

Zygospore formation by *Phycomyces blakesleeanus* is not inhibited at 7° (Hawker *et al.* 1957). Segments of this fungus used as a source colony, without contact, induced initiation and maturation of a number of zygospores in young colonies of *Rhizopus sexualis* at that temperature.

Slide cultures of *Rhizopus sexualis* supported on glass rods over older agar plate cultures of the same or other species also produced a few zygospores at low temperature.

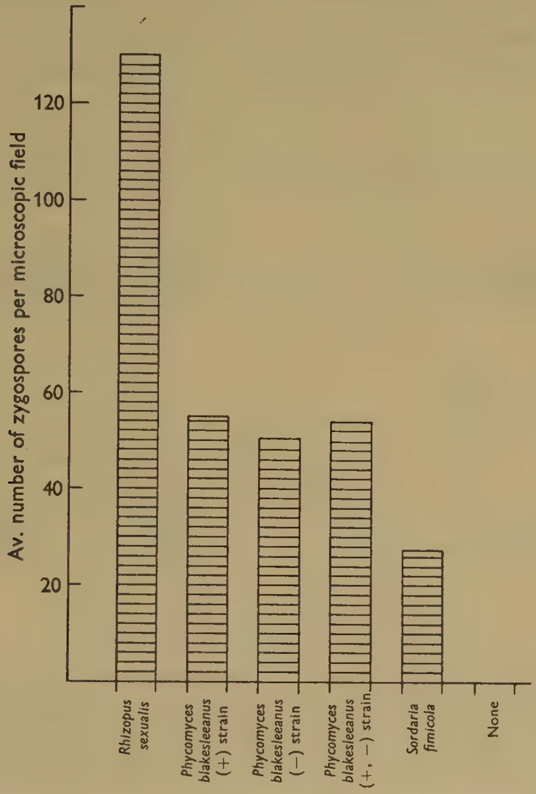

Fig. 2. The effect of volatile products of living fungi on zygospore production by *R. sexualis*. Each block represents the number of zygospores produced by *R. sexualis* in the presence of volatile substances from living cultures of the fungi indicated at base of diagram.

Stimulatory effects also occurred at a temperature of 20° which is near the optimum for *Rhizpous sexualis*. Figure 2 shows the number of zygospores in slide cultures supported over living cultures of *R. sexualis*, *Phycomyces blakesleeanus* and a Pyrenomycete (*Sordaria fimicola*) and in control slide cultures (supported over sterile plates of agar). In control cultures a number of aerial hyphae (stolons) developed before any zygophores were produced, but in treated cultures the first-formed aerial hyphae were zygophores. Zygospore production was stimulated equally by single or by paired compatible strains of the heterothallic *P. blakesleeanus*. In later experiments other sporing species of the Mucorales were tested and gave similar results. None was as effective as *R. sexualis* itself, but all were more effective than the unrelated Sordaria.

Rhizoctonia solani, a non-sporing fungus, was also tested as a source. Slide cultures of *Rhizopus sexualis* grown in the presence of *Rhizoctonia solani* showed no increase in zygospore production as compared with control cultures which had no source culture present.

Effects of a volatile stimulatory substance produced by mature colonies. Glass cylinders, 2 cm. deep and 3 cm. in diameter, with two glass tubes 3 mm. in diameter, fused into opposite sides as near the top as possible, were sealed by glass plates stuck on to the lower and upper surfaces with silicone grease (Fig. 3). The tubes were plugged with cotton wool and the units could then be autoclaved. The upper glass plate was temporarily removed and 10 ml. of molten sterile malt agar medium were allowed to solidify on the bottom of the chamber. A suspension of sporangiospores was then poured over the agar medium and the chamber was rocked until the suspension was evenly distributed over the surface of the agar, after which the glass lid was finally replaced. The inoculated units could be connected together and an air stream passed through the series. The series could be arranged so that the air stream passed over mature cultures (usually growing in large flasks) as *source cultures*, before passing over young ones (*test cultures*); the cultures could be maintained at different temperatures so that the air passed from cultures at optimum temperatures over others at low temperatures; the effluent from mature cultures could be scrubbed by passage through various solvents to remove metabolic products likely to be present, or known gases could be passed through the system. The test cultures could be conveniently examined *in situ*.

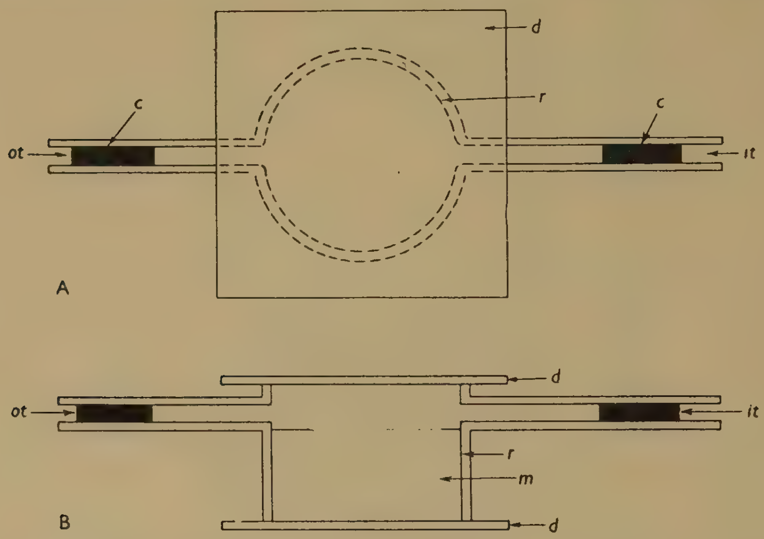

Fig. 3. Vessel designed to allow flow of gas over culture. A, plan and B, elevation of culture vessels used in experiments to test effect of volatile substances from mature cultures on zygospore production of *R. sexualis*. *r* = glass ring, *it* = inlet tube, *ot* = outlet tube, *c*, cotton-wool plug, *d* = glass disk sealed to ring (see text), *m* = agar culture medium.

In all experiments the source cultures were grown for 48 hr. at 20°, by which time zygospores were numerous, before being used. Test cultures were grown at

20° for 24 hr., by which time a few young zygospores but no mature ones had developed, or for 48 hr. before being treated. Air was then passed from source to test cultures. In the first few experiments the air was passed in continuous flow, but in later ones the total gas space of the system was determined and the air supply was controlled manually in such a way that the air over the source cultures was moved on over the test ones every 4 hr., thus allowing a longer period for any volatile product to accumulate over the source colonies and later for it to influence the test colonies. Moreover, this method avoided differences due to slight irregularities in flow rate. The results were more consistent than those with continuous flow but otherwise confirmed them. Control cultures received air which had passed over uninoculated culture medium.

The results of three typical experiments are shown in Table 1. In the first and second of these both source and test cultures were maintained at 20° and treatment was commenced 24 hr. after inoculation of the test culture. The rate of zygospore production and maturation was greater in the treated than in the control cultures. In the third experiment the test cultures were transferred to 7° and treatment was begun 48 hr. after inoculation. The number of new zygospores initiated and the number of young ones maturing was greater in the treated than in the control cultures. In contrast the number of sporangia was greater in the controls. In a further experiment in which both source and test cultures were transferred to 7° 48 hr. after inoculation there was no stimulatory effect of the source upon the test one. Under these conditions it is likely that the rate of metabolism of the cultures would be so slow that the amount of volatile stimulatory substance produced by the source culture would be negligible, or that the substances would pass over too quickly for the test colonies to use it.

Table 1. *The effect on zygospore production at* 20° *and at* 7° *of a stream of air which has previously passed over a mature culture growing at* 20°

Age of test culture at commencement of treatment	Temperature at which test culture was treated	Treatment of test cultures*	No. of replicates (10 fields counted in each)	Av. no. of zygospores per microscopic field
24 hr.	20°	Treated	9	75
		Control	6	51
		Treated	8	17†
		Control	12	6†
48 hr.	7°	Treated	6	10
		Control	6	7

* Treated and control cultures received air which had previously passed over mature source cultures and over uninoculated culture media, respectively.

† Only mature zygospores counted in this experiment.

It is thus clear that some volatile product of metabolism stimulated zygospore development and that an adequate supply of it from external sources counteracted the inhibitory effect of low temperature. One must conclude that this inhibitory effect was at least partially due to failure of the fungus to synthesize the unknown volatile product at the low temperature.

Possible nature of the volatile stimulatory substance. The observation that cultures of *Rhizoctonia solani* do not produce volatile substances capable of stimulating zygospore production by Rhizopus indicates that the active factor is unlikely to be a common metabolic product, such as carbon dioxide, ammonia or ethanol. Moreover, no increase in zygospore production resulted when carbon dioxide or ammonia, at concentrations permitting vegetative growth, were passed over a young culture in a chamber of the type described in the previous section. The removal of carbon dioxide or ammonia, by passage through traps placed between source and test cultures and containing 20% aqueous potassium hydroxide or Nessler's reagent, respectively, did not alter the activity of the effluent. Ethanol (70% aqueous) placed between source and test cultures also failed to influence zygospore formation. Thus it is clear that none of these substances is the cause of the stimulatory effect.

Passage of the effluent through dilute sulphuric acid removed the activity, even when the humidity was restored by subsequent passage through water. This indicates that the substance is probably basic. Titration of the acid against 0·01 N-NaOH, however, did not show any change in the titration value; any substance removed by the acid must have been present in very small quantities.

Hepden & Folkes (1960), in considering the possible relationship between nucleic acid metabolism and the initiation of zygospores, suggest that the volatile substance is likely to be a methyl donor. They discuss the possibility that this might be methylamine and outline a probable mechanism for its action. The removal of the stimulatory effect of gaseous effluent from mature cultures of Rhizopus by passage through acid is consistent with methylamine being at least partially effective. Accordingly a number of methyl donors and other methylated compounds were tested for their effects on *Rhizopus sexualis*.

In some experiments these substances were incorporated in glucose-asparagine agar, glucose-ammonium salts agar or 1% malt agar, to which they were added when they were cooling. In others compounds were placed on disks of filter paper which were laid directly on the agar plate or were placed on sterile glass coverslips which were then laid on the medium. In another series of experiments, *Rhizopus sexualis* was grown on glass microscope slides in a film of liquid culture medium. The non-volatile substances to be tested were added to this liquid; the volatile ones were placed in Petri dishes and the test slide cultures were supported above them. Both plate and slide cultures were tested at 20° and at 7°, to which they were transferred after 24 hr. at 20°. The results of all these experiments are collected in Table 2.

All the methyl donors tested were either ineffective or were more or less toxic even at low concentrations. None of them induced an increase in zygospore initiation and maturation comparable to that shown in Table 1 as resulting from the volatile substances produced by mature cultures. Choline, methylamine, DL-serine, formaldehyde, formic acid and methanol showed some counteraction of the effect of low temperature on cultures which had already begun to form zygospores before transfer to 7°. None of these substances, however, significantly increased the number of zygospores developing in cultures which, at the time of transfer to low temperature, bore no mature zygospores. In all cultures there was an increase in the number of pseudophores which, as already pointed out, have been interpreted as potential progametangia and which, under suitable conditions, sometimes develop into azygospores. Callen (1940) reported an increase in the number of pseudophores produced by *Rhizopus sexualis* in the presence of the related *R. nigricans*. It is thus possible that the production of pseudophores indicates activity comparable to that of the naturally produced volatile substance, but that the beneficial effects are counteracted by the toxic nature of the pure compounds. Concentrations of these pure substances too low to be toxic had no effect on the production of pseudophores or of normal zygospores. These results do not exclude the possibility that the active substance is methylamine or some other methyl-donor.

Table 2. *Effects of various methyl donors and methylated compounds on growth and zygospore formation at* 20° *and at* 7–10° *after* 24 *hr. at* 20°

			Effects at 20°		Effects at 7–10°	
Substance	Percentage concentration	Method of supply	On growth	On zygospore formation	On growth	On zygospore formation
Methionine	0·1–0·2	A	None[1]	None	+	None
Methylamine	7·0–70·0	B, C D	−, p	None	±	p
Methylamine. HCl	0·1	A	−	None	±	p
Methanol	10–100	C, D	−	p	None	p
Thymine	0·1	A	None	None	None	None
Choline chloride	0·001–1·0	A	±	None	±	+ +
Formaldehyde	0·08–0·64	A, D	−	−[2]	−	p
Formic acid	0·09–0·46	B, D	−	p	−	p
Glycine	0·01–0·1	A	None	None	None	None
DL-Serine	0·01–0·1	A	+	+	None	+ +
Betaine. HCl	0·01–0·1	A	None	None	None	None

Key to Table 2

A, incorporated in culture medium.
B, on paper disk laid on agar medium.
C, on glass coverslip laid on agar medium.
D, on dish with slide culture supported above.
+, slight increase.
+ +, increase.
−, decrease.
±, slight increase or decrease according to concentration.
p, increase in number of pseudophores or of irregular swellings resembling pseudophores.
[1], slight increase in absence of other source of nitrogen.
[2], pseudophores developed in slide cultures only.

DISCUSSION

The experiments show that a volatile substance or substances produced by mature cultures of *Rhizopus sexualis* stimulates zygospore production by young cultures of the same fungus at optimal temperatures (*c.* 20°) and partially counteracts the inhibitory effects of low temperatures (7–10°). It is established that the active factor in the effluent gases is not carbon dioxide, ammonia or ethanol, that it is basic and probably produced in extremely small quantities. The identity of the active substance has not been established but the experimental results are not inconsistent with its being methylamine. It is clear, however, that the effects of the naturally produced substance cannot be exactly reproduced by low concentrations of pure methylamine.

The effects of the presence of cultures of related species were less than those of mature cultures of *Rhizopus sexualis* itself, and the unrelated *Sordaria* was even less effective. It is possible, therefore, that the activity of the effluent from mature cultures is not due to a single volatile substance but to a mixture of several such substances. The exact composition and proportions of such a mixture might well vary with different species, thus accounting for the high degree of specificity observed and the decreasing effect on *R. sexualis* with decrease in the relationship between test and source cultures. It is thus unlikely that volatile substances stimulating other fungi are exactly identical with that produced by *R. sexualis*. Of such substances, an unidentified one described by Banbury (1954) as stimulating zygotropism in *Mucor hiemalis* might conceivably be of similar nature; one described by McTeague, Hutchinson & Reed (1959) as stimulating growth of germ tubes of *Agaricus campestris* and identified as 2-3-dimethylpentene is unlikely to resemble closely that produced by *R. sexualis*. It is hoped that further work now in progress may establish the nature of the latter and elucidate the part that it plays in zygospore initiation.

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Toxicity of the Extracellular Phase of *Prymnesium parvum* Cultures

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SUMMARY

Cytotoxic material ('prymnesin') excreted into the growth medium by the phytoflagellate *Prymnesium parvum* was partially purified and crudely characterized. It proved to be a highly potent haemolysin; its solubility properties and some other characteristics are those of a lipid, perhaps a saponin. The high haemolytic activity which characterized the toxin was often also accompanied by a high ichthyotoxic activity. A method of locating haemolytic activity in a paper chromatogram is described. Fish were killed not by prymnesin alone, but were rendered highly susceptible to prymnesin poisoning by a range of cofactors: streptomycin sulphate, calcium chloride, magnesium chloride. Ichthyotoxicity in cofactor + prymnesin systems conformed within a wide concentration range to the relationship: $a \times b = Q$, where a is a prymnesin concentration, b a minimum concentration of cofactor which elicits a lethal effect in presence of a, and Q is a cofactor-specific constant. The recognition of this relationship has enabled a sensitive assay to be developed. Practical implications are discussed and the hypothesis proposed that prymnesin acts on fish by inducing a state of abnormal permeability of the gill.

INTRODUCTION

Outbreaks of mass mortality among gill-breathing inhabitants of pond waters have been associated with blooms of a toxigenic phytoflagellate *Prymnesium parvum* Carter (Otterström & Steeman-Nielsen, 1940; Reich & Aschner, 1947; Shilo & Aschner, 1953; Yariv, 1955, 1958; Shilo & Rosenberger, 1960). Severe losses are inflicted on the carp industry of Israel by this agent. The present communication describes a procedure for the recovery of stable and partially purified toxic material 'prymnesin' from the culture fluid of *P. parvum* cultures, describes some general properties of the preparation and discusses methods for its assay.

METHODS

Cultivation of Prymnesium parvum. Pure cultures were kindly made available to us by Mrs Mira Shilo. Strain no. 1 was originally isolated in Israel from fish ponds by Reich & Kahn (1954) and strain no. 2 by Droop (1954) in Scotland from supralittoral pools.

Pure cultures were made in a medium composed of 0·02 % (w/v) liver extract (Difco), 0·02 % (w/v) Bacto-tryptone (Difco) and 10 % (w/v) sea water in distilled water in 500 ml. conical glass flasks maintained under fluorescent lamps at 20°.

A 2-week-old culture was used in the proportion of 1:10 for inoculum. Large-scale preparations were made from samples of pond water (batch size about 50 l.) which had been transferred during prymnesium bloom to a tank which was maintained at ambient temperature (10–25°) under fluorescent lighting for 1 to 2 weeks, in which time the toxic activity reached its peak.

Detection and measurement of haemolytic activity

Reference standard. Digitonin (Merck, C. P.) served as a standard for reference; the stock solution was at a concentration of 0·026 % (w/v) in water. At a dilution of 1/200 in isotonic sodium chloride, this effected 50 % lysis of bovine erythrocytes within 45 min. in conditions of a standard assay described below.

Preparation of samples for assay. Samples to be assayed were brought to pH 5·0 and to isotonic salt concentrations. Extracts of dry prymnesin preparations were prepared by either of the following methods. (*a*) Sample (about 2 mg.) was moistened with a drop of 0·1 M-sodium hydroxide and suspended in 10 ml. water. The suspension was cleared by centrifugation; the supernatant fluid was immediately brought to pH 3–4 with dilute hydrochloric acid, whilst the sediment was extracted again as before. The extracts were pooled, the pH adjusted and brought to a known total volume and to appropriate salt concentration. (*b*) Sample was stirred with successive portions of methanol until all the haemolytic activity had been taken into solution. Extracts in methanol were diluted at least 1/100 with buffered saline before assay.

Standard assay system. Since prymnesin often lost activity rapidly at pH > 5 (see Results), assays were performed in acidified blood. As bovine erythrocytes withstand acid conditions better than do sheep erythrocytes, they were used in preference to the latter. The blood was taken into an equal volume of isotonic salt solution (0·073 M-sodium chloride, 0·042 M-sodium citrate, 0·114 M-glucose; pH 6·1) and stored at 4°. Before assay, stored red cells were washed 3 times in the cold with 10 vol. of buffered saline (0·130 M-sodium chloride adjusted to pH 5·0 with 0·020 M-citrate). The assay system consisting of toxin with approximately 0·4 % (v/v) bovine erythrocytes in buffered saline at pH 5·0 was incubated for 45 min. at 35 ± 0·5°.

The degree (%) of haemolysis was determined colorimetrically, essentially as described by Collier (1951). The values of optical density were respectively 340 and 190 (galvanometer units measured in a Klett galvanometer with filter 54) for 0 and 100 % haemolysis. The decrease of optical density was closely proportional to the % haemolysis at all values of the latter.

A unit of haemolytic activity was defined as that amount of prymnesin which effected 50 % lysis of bovine erythrocytes in one ml. of standard assay system. This unit is equivalent to 1·3 μg. digitonin. Figure 1 shows that in the range 0·8–1·2 units there was an almost linear relationship between concentration of haemolysin and % haemolysis. Titration within an accuracy of ± 7 % generally required 30 units toxin in a solution at a concentration of at least 3 units/ml.

Test of haemolytic activity on paper. Loci of haemolytic activity were detected in paper chromatograms by means of an erythrocyte spray (2 %, v/v, bovine erythrocytes in 0·137 M-sodium chloride + 0·020 M-sodium phosphate; pH 6·8). This was applied to the vertically suspended paper from a horizontal direction, starting from the top and working downwards in such a way as to leave a dry area at the top of the paper strip while excess of fluid accumulated at the bottom. While the strip dried in air (10–20 min.), fluid flowed through it in an upward direction. Whilst such a flow did not cause migration of erythrocytes, any haemoglobin released by haemolysis was moved by the current and formed a dark spot in the region above the haemolytic locus; a bleached area remained below the dark spot. Certain non-haemolytic hydrophobic substances caused the erythrocytes to aggregate with attendant whitening or darkening of the locus (Table 1). However, as such changes were not accompanied by migration of haemoglobin, they were readily distinguished from a true haemolytic action. A reaction observed by Heftman & Hayden (1952) at sapogenin loci on paper is believed by us to have been of this non-specific kind and to have been interpreted erroneously as a sign of haemolytic activity.

Table 1. *Specificity of the haemolysis reaction on paper*

Substance*	Solvent	Reaction†	Minimum reactive dose (μg.)
Digitonin	Methanol	*h*	0·8
Prymnesin‡	*n*-Propanol + water (1 + 1, v/v)	*h*	1·5
Lysolecithin	Water	*h*	5
Saponin	Methanol	*h*	40
Digitogenin	Methanol	*nh*	8
Cholesterol	Methanol	*nh*	8
Kryptogenin acetate	Ethanol + chloroform (1 + 1, v/v)	*nh*	16
Tristearin	Ethyl ether	*nh*	100

* Lysolecithin was kindly supplied by Mr G. Rimon. Digitogenin and kryptogenin acetate were made available through the courtesy of Professor C. Djerassi.

† A haemolytic reaction is designated *h* and a non-specific colour change by *nh*.

‡ Fraction Xb (see text) was used for this experiment.

Measurement of ichthyotoxic activity

Fish. Gambusia minnows (25–40 mm. long) collected from pools in Beisan Valley, Israel, served as the test organism. The animals were maintained in the laboratory in tanks of tap water under a gentle stream of air.

Standard assay system. Ichthyotoxicity was measured on the basis of the ability of prymnesin to enhance the susceptibility of fish to the lethal action of streptomycin. Groups consisting of five fish were immersed in beakers at 20° into 20 ml. of solution containing prymnesin, sodium bicarbonate at 5 mM and streptomycin sulphate at 2·0 mM (0·1 %) in distilled de-ionized water. The solution was adjusted to pH 8·0 with NaOH and held within ± 0·2 pH units throughout the assay. The 'fish unit' of toxin was defined as that minimum amount of prymnesin/ml. which killed all five minnows present in 20 ml. of standard solution containing streptomycin sulphate at pH 8·0 during 3 hr. at 20°. In the conditions of this assay, streptomycin sulphate alone or prymnesin alone did not kill any of the fish. The titrations of toxicity were reproducible within ± 10 %. A total of about 100 fish was generally used per titration.

Ichthyotoxin preparation. Except as otherwise indicated, prymnesin at stage Z of purification (see under Results) was used in experiments on ichthyotoxicity. Stock solutions (pH 4·0) in water were stored at 6°.

RESULTS

Supernatant fluids from pure cultures of *Prymnesium parvum* increased in toxicity during the first 2 weeks. Haemolytic titres reached 6–30 units/ml. and ichthyotoxic titres about 6 units/ml.

Purification of toxic principles

A pure culture (strain no. 1) of *Prymnesium parvum* with an activity of 16 haemolytic units/ml. at 16 days was cleared by centrifugation (12,000 rev./min. in a Sharples centrifuge) and the culture fluid brought to pH 10–11 with conc. sodium hydroxide, added with vigorous stirring. A flocculent precipitate, consisting mainly of magnesium hydroxide, was allowed to sediment (1 hr.), packed down by centrifugation and dissolved in N-hydrochloric acid (100 ml.). The solution (pH 2–3) was dialysed against 0·1 mM-hydrochloric acid through cellophan at 6° for 3 days. On freeze-drying the material remaining in the dialysis sac (dialysand) 140 mg. of a fluffy, somewhat hygroscopic powder (Z) was obtained. About 50 % of the original haemolytic activity was recovered in this fraction. Its haemolytic and ichthyotoxic activity remained unchanged during prolonged storage (1 year at −15° in an evacuated ampoule). Product Z was converted into a granular product (Y) by grinding under cold absolute acetone. Solution of this material in 50 ml. methanol gave on evaporation under reduced pressure 12·5 mg. of a slightly yellow powder (X) having an activity of 4800 haemolytic units/mg. A large-scale operation conducted on a monoalgal rather than pure culture is summarized in Table 2.

Table 2. *Purification and resolution of active principle in supernatant fluid of monoalgal* Prymnesium parvum *culture*

Purification procedure was essentially as that described in the experiment with a pure culture (see text).

Fraction*	Recovery of haemolytic activity (% of starting material)	Dry weight (mg.)	Haemolytic activity (units/mg.)	Ichthyotoxic activity (units/mg.)
Culture fluid	100	10^6	0·5	0·15
Z	78	299	1240	400
Y	78	204	1640	530
X	78	52	6700	1900
Xa	6	4	6400	—
Xb	68	44	6700	1700

* Letter symbols are used to designate successive steps in purification as described in the text.

Resolution of toxin in a propanol+water system. Product X was washed in absolute *n*-propanol (20 ml.) without loss of activity and then dispersed in *n*-propanol+water (90+10, v/v). About 11 % (fraction Xa) of the total activity was taken into solution by this solvent. The residue was dispersed in 30 ml. of *n*-propanol+water system (50+50, v/v), to afford a solution which was cleared by centrifugation and then concentrated under reduced pressure with successive additions of absolute *n*-propanol. On evaporation of the solvent, 7·4 mg. of a light yellow powder (Xb) were recovered. This contained most of the original activity at

5600 haemolytic units/mg., a value nearly eight times that found with digitonin in the standard assay conditions.

Chromatographic resolution of toxin on cellulose. Suitable solvent systems applied on a paper sheet (Table 3) or on a cellulose column (Fig. 2) resolved the activity of product Z into at least two components. An all-glass column (600 mm. long and 35 mm. in diameter) contained Whatman's Ashless Cellulose Powder pretreated as recommended by Stoll & Kreis (1951). The developing system was ethyl acetate + *n*-propanol + water (8 + 2 + 3, v/v). Fraction Z of prymnesin (50,000 units contained in 60 mg.) was applied in the aqueous phase of the solvent and developed in the organic phase. Successive 4·0 ml. fractions of effluent were examined for activity > 360 units/ml. by paper test. A fast-moving fraction (corresponding to Xa in Table 2) represented 20 % of the total activity put in the column; a slow-moving fraction (corresponding to Xb in Table 2) represented ⩾ 60 % of the total activity

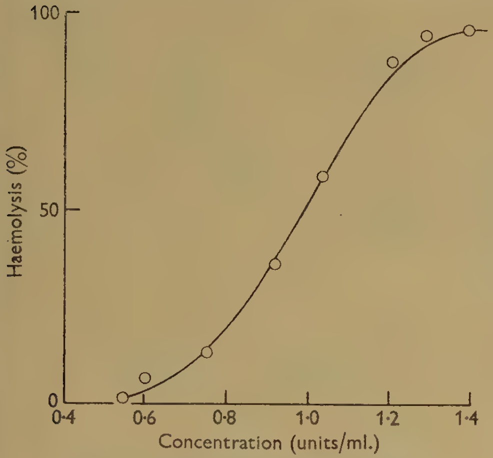

Fig. 1. Concentration effect function of prymnesin.

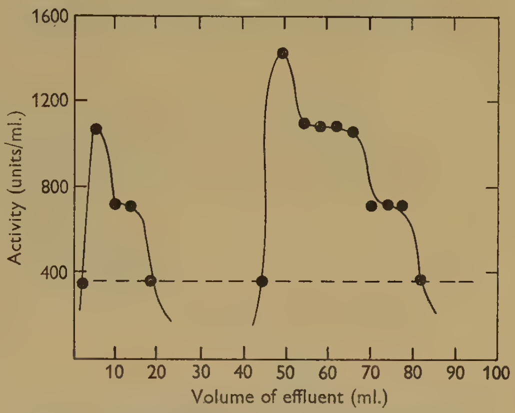

Fig. 2. Chromatographic resolution of prymnesin on a cellulose column.

Table 3. *Mobility of various haemolysins on paper*

Whatman no. 1 paper was used with a development time of 15 hr. at room temperature.

Solvent system (v/v)	Substance	Appropriate R_f*
n-Amyl alcohol + acetic acid + water (7 + 3 + 10)	Prymnesin	**0·06**; 0·17
n-Butanol + water (1 + 1)	Prymnesin	0·18
Ethyl acetate + *n*-propanol + water (8 + 2 + 3)	Prymnesin	**0·21**; 0·34†
n-Amyl alcohol + *n*-butanol + water (1 + 4 + 5)	Prymnesin	0·45
Ethyl acetate + *n*-propanol + water (7 + 5 + 10)	Prymnesin	0·90
n-Butanol saturated with water	Lysolecithin	0·68
	Digitonin	0·05
	Prymnesin	0·05

* Where toxin was resolved into components, the R_f value of the major component is given in bold figures.

† Ninhydrin-positive substances (test performed as by Berry & Cain, 1949) in the sample were resolved by this system and gave R_f values of 0·00, 0·06, 0·15. At the two haemolytic loci, however, no substance which gave a positive reaction with ninhydrin was detected.

applied to the column. The furfural test (Feigl, 1954) for carbohydrate in both these fractions was positive. The minimum haemolytic activity which was detectable by the analytical procedure used is indicated in the figure by the broken horizontal line. A major (slow) component in product Z had the same R_f value as the active compound found in the Xb fraction described above. The R_f value of the minor (fast) component of product Z corresponded to the active compound in the Xa fraction.

General properties of toxin

Some solubility properties of the prymnesin are given in Table 4. Information about keeping qualities of this preparation, and on behaviour with precipitating agents and adsorbents as well as miscellaneous other features, is collected in Table 5.

Table 4. *Solubility of prymnesium haemolysin in organic liquids*

A sample (2 mg.) of prymnesin purification Z was stirred with 0·2 ml. solvent, centrifuged, and the supernatant fluid then tested for haemolytic activity.

Haemolytic activity	
1000 units or more/ml.	200 units or less/ml.
Methanol, pyridine*, *n*-butanol (sat. with water), *n*-propanol + water (1 + 1, v/v), *e*-systems†.	*n*-Propanol, *n*-butanol, acetone, diethyl ether, light petroleum, ethyl acetate, ethyl acetate (sat. as to water), benzene, chloroform, carbon tetrachloride.

* Activity in pyridine declined rapidly.

† *e*-systems = aqueous and organic phase of ethyl acetate + *n*-propanol + water (8 + 2 + 3, v/v).

Prymnesin solutions (10–100 units/ml.) in water showed a marked decrease in titre when kept for 60 min. at 35°. When solutions which had been partially inactivated by such treatment were subsequently brought to pH 4·0, their original activity was rapidly restored.

Relation of ichthyotoxic and haemolytic activities. Though the ratio of haemolytic activity to ichthyotoxic activity in a given preparation of prymnesin remained roughly constant throughout purification (Tables 2, 5), wide variations in ratio were encountered between different batches of toxin. Several preparations which exhibited high haemolytic activity indeed had no apparent ichthyotoxicity.

Observations on ichthyotoxicity of water samples from pure cultures and from ponds during prymnesium bloom. As reported by Liebert & Deerns (1920), Otterstrøm & Steeman-Nielsen (1940) and Reich & Aschner (1947), samples of water taken from ponds during prymnesium bloom were frequently but not invariably ichthyotoxic. When toxic samples were dialysed through cellophan (72 hr.; 6°; pH 4·0), the separated dialysate and dialysand had no lethal effect on fish, but were again toxic when pooled.

Passage through a cation-exchange column (H^+ form of Amberlite IRC 50) abolished the ichthyotoxicity of pond-water samples. On elution of the column with N-HCl, material was recovered which conferred toxicity on dialysand prepared from a toxic water sample. The active factor in the eluate was not destroyed by ashing. Dialysand of toxic water samples formed a toxic system

Table 5. *Influence of various treatments on cytotoxic activities of prymnesin*

Aqueous solutions contained 10–100 units prymnesin/ml.; solutions in organic liquids represented extracts of product Z at a concentration of about 1000 units/ml. The pH values of the aqueous solutions were adjusted with HCl to pH 0·0, 4·0 and 6·0 and with $NaHCO_3$ to 8·0. Except as otherwise stated, all treatments tabulated below were performed at 25° and lasted for 5 min.

Treatment	Tested property or reagent	Solvent	Particulars of treatment	Recovery of original activity (%) in	
				Liquid phase	Solid phase[2]
1	Storage	Water	700 hr.; 6°; pH 4	**100**[1]	*
2	Storage	Water	24 hr.; pH 4	**100**	*
3	Storage	Water	24 hr.; pH 8	0	*
4	Thermostability	Water	15 min.; 98°; sealed vessel; pH 4	**100**	*
5	Thermostability	Water	15 min.; 98° (boiling); pH 4	0	*
6	Thermostability	*n*-Propanol	15 min.; 94° (boiling)	100	*
7	Thermostability	Methanol	15 min.; 62° (boiling)	100	*
8	Hot acid	Water	15 min.; 98°, pH 0	0	0[3]
9	Water/gas interphase	Water	24 hr.; stream of N_2; pH 4	**60**	*
10	Diffusibility	Water	72 hr.; 6°; cellophan membrane; pH 4	**100**[4]	*
11	Cellulose	Water	Paper (Whatman no. 1); pH 4	30	70[5]
12	Ca phosphate	Water	15 min.; Na-acetate 5 mM; pH 4	**44**[6]	—
13	$Mg(OH)_2$	Water	0·1 M-$MgCl_2$; initial pH 4 brought to pH 11	0	57[7]
14	$(NH_4)_2SO_4$	Water	6°; 0·24 of sat.; pH 6	0	34[8]
15	Chloroform	Water	6°; shaking; pH 4	0[9]	32[10]
16	Cholesterol, acetone or *n*-amyl alcohol	Methanol	Reagent at 1 %	60 to 80	—[11]
17	Diethyl ether	Methanol	Reagent at 1+1 (v/v)	36	42[12]
18	Petroleum ether	*e*-System[13]	Reagent at 1+1 (v/v)	30	56[12]

[1] Values denoted by bold figures designate solutions that were tested also as to ichthyotoxic activity. In all these cases, ratio of haemolytic to ichthyotoxic activity was found not to have been altered significantly by the treatment in question.

[2] Absence of precipitate formation is indicated by asterisk.

[3] The precipitate was soluble in benzene.

[4] The dialysate contained no activity.

[5] Methanol served as eluent.

[6] One ml. gel suspension (prepared according to Singer & Kearney, 1950) was added to 10 ml. prymnesin solution. At pH 7–10, little or no activity was adsorbed by this reagent.

[7] Precipitate, consisting mostly of $Mg(OH)_2$, was dissolved at pH 2 (adjusted with HCl) and dialysed before titration.

[8] The solid, being of low specific gravity, floated when the suspension was centrifuged; it was dissolved in water.

[9] Material in solution in the aqueous and in the organic phase of the solvent system was inactive.

[10] The precipitate was collected at the chloroform–water boundary and dissolved in water after alkali treatment.

[11] Precipitate formed with 1 % reagent was not visibly dissolved and failed to release any activity into either methanol or 0·01 M-NaOH in water. Increase of the concentration of reagent by several fold failed to elicit any further precipitation.

[12] After removal of this precipitate, increase of the ether concentration to 2 vol. gave no further precipitate, but at 3 vol. a second precipitate formed with attendant disappearance of all activity from solution. The activity recovered on solution of the second precipitate into methanol represented 40–70 % that in the original supernatant fluid.

[13] *e*-system = organic phase of ethyl acetate + *n*-propanol + water (8 + 2 + 3, v/v).

when it was mixed with 10 mM-calcium chloride, 10 mM-magnesium chloride or 2 mM-streptomycin sulphate. In absence of dialysand the same solutions were innocuous to fish. Chlorides of monovalent cations (Na, K) at 20 mM were not toxic to the fish either in the presence or absence of dialysand. Similar results were also found when dialysed centrifugates from pure cultures of *Prymnesium parvum* were used for these experiments in the place of the pond-water dialysands.

Effect of prymnesin on susceptibility of fish to poisoning by various compounds

Toxicities of various inorganic salts and of streptomycin sulphate in the presence and absence of prymnesin are compared in Table 6.

The manner in which LD100 of selected compounds varied with the prymnesin concentration is shown by Fig. 3. These data fit a simple relationship: $a \times b = Q$, where a is prymnesin concentration, b is the minimum concentration of cofactor which elicits a lethal effect in the presence of a, and Q is a cofactor-specific constant whose value defines the LD100 of the cofactor for systems in which prymnesin is at unit concentration.

Cofactors all had practically the same LD100 values when measured in absence of prymnesin on prymnesin-pretreated minnows as when measured in the presence of prymnesin (see Table 7). The time lapse between the beginning of exposure to cofactor and the onset of death greatly exceeded the duration of the previous immersion of the fish in the prymnesin solution. This implies that the survival times of fish in the cofactor + prymnesin systems probably depended on the time required for the action of cofactor on the prymnesin-sensitized fish, rather than on the time during which the sensitization by prymnesin was accomplished.

Table 6. *Influence of prymnesin on ichthyotoxicity of various compounds*

Compound	Buffer (5 mM; pH 8·0)	LD100 (mM) of compound in absence of prymnesin	LD100 (mM) of compound in presence of prymnesin (1·0 units/ml.)
$CaCl_2$	$NaHCO_3$	60	1
Streptomycin SO_4	$NaHCO_3$	20	2
$CaSO_4$	$NaHCO_3$	> 20	5
$MgCl_2$	$NaHCO_3$	80	5
$MgCl_2$	Na_2HPO_4	> 40	20
KCl	Na_2HPO_4	> 20	—
NaCl	Na_2HPO_4	> 50	—

The LD100 value of prymnesin in the standard assay system was doubled when a solution of prymnesin (20 units/ml.) was held for 6 hr. at room temperature at about pH 10 before the test was carried out. The solution, thus partially inactivated, was rapidly restored to its original titre by subsequent incubation at pH 4·0. Thus there appeared to be at least a qualitative resemblance between the effects of successive exposure to alkali and acid on ichthyotoxicity and haemolytic activity, respectively.

To learn whether plant saponins require cofactor for the manifestation of the lethal effect on fish, methanol-soluble components of a commercial saponin preparation (British Drug Houses Ltd.) were taken into water and dialysed against dilute acid (10^{-4} N-hydrochloric acid for 3 days). The LD100 values of the dialysand at pH 6·0, 7·0 and 8·0, respectively (pH adjusted with 0·01 M-sodium phosphate), were as follows (in μg. original crude preparation/ml. final system): 250, 167, 125. Addition of cofactor (20 mM-$MgCl_2$) to such dialysands did not alter their LD100 values.

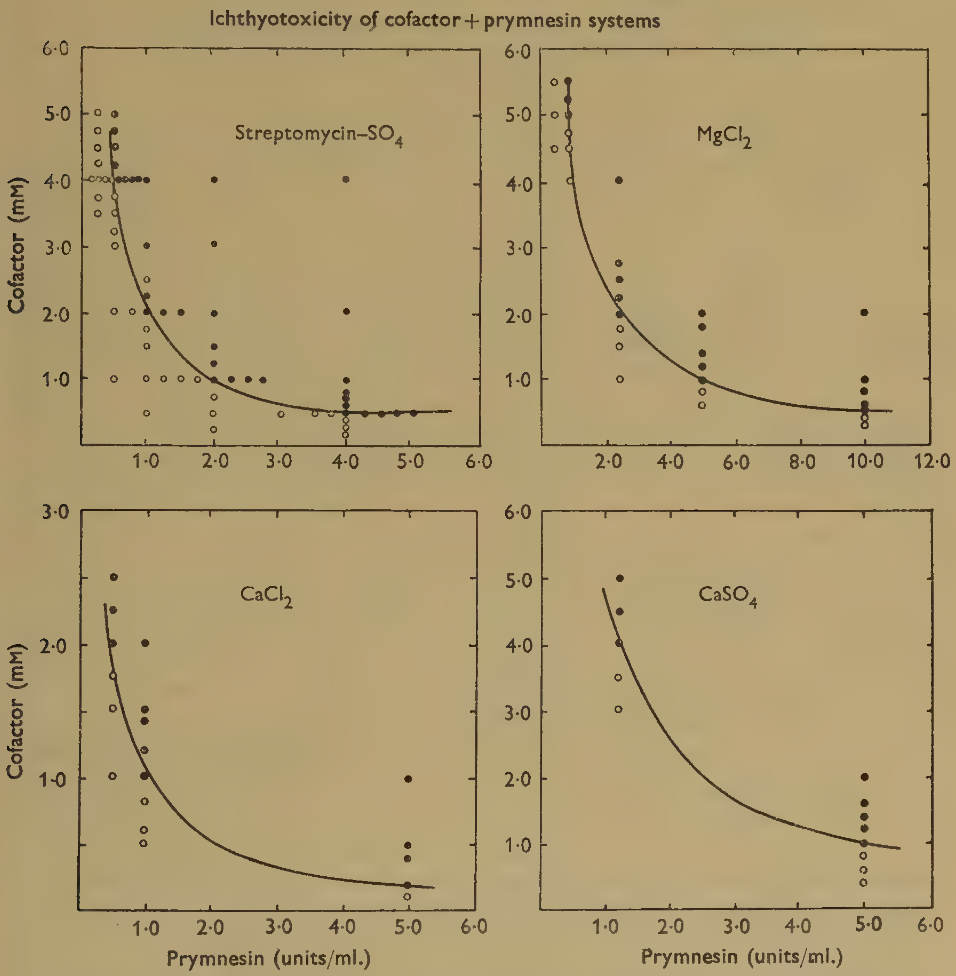

Fig. 3. Ichthyotoxicity of cofactor + prymnesin systems. Test mixtures contained in each case prymnesin and a single added salt under the standard conditions. Fish were exposed to prymnesin at graded dilutions in the presence of a fixed cofactor concentration and to cofactor at graded dilutions in presence of a fixed concentration of prymnesin. Solution mixtures in which one or more of the animals survived for 3 hr. are recorded by ○ and those in which no animal survived by ●. Drawn curves are calculated from equation (1) on the basis of appropriate values assigned to Q (streptomycin sulphate, 2·0; magnesium chloride, 5·0; calcium chloride, 1·0; calcium sulphate, 5·0), concentration of cofactor being expressed in mM and that of prymnesin in units/ml. of final system.

Table 7. *Effect of prymnesin on the susceptibility of fish to streptomycin sulphate*

Minnows were immersed in prymnesin solution and then transferred to solution containing streptomycin sulphate but no prymnesin.

Treatment with prymnesin		Concentration of streptomycin sulphate (mM)			
		1	2	3	4
		Max. survival time (min.)			
Units/ml.	Duration (min.)				
1·5	5	180	110	82	67
	15	180	90	65	59
3·0	5	.	57	.	.
	15	.	55	.	.
5·0	5	60	38	.	.
	15	64	35	.	.

DISCUSSION

A simple diagnostic procedure to indicate whether prymnesin was the causal agent in any particular outbreak of mass mortality among fish has been sought. Some properties of prymnesin which are disclosed in this paper might be useful in this connexion. They are in particular the non-dialysability of this agent, its rather unusual solubility pattern and its dependence for action after dialysis on an added cofactor. Solubility in methanol and in *n*-propanol water solvent systems distinguishes the toxic material in prymnesin from simple proteins and polysaccharides. The observed properties are consistent with the view that the toxic material is lipid and in which there are present both non-polar and polar moieties, both of which contribute significantly to the over-all behaviour. It has also been noted that the ratio of haemolytic to ichthyotoxic titre in a preparation made from a given starting material tends to maintain nearly a constant value throughout successive stages in purification, but that a wide variation in the activity ratio can be observed as between preparations made from different batches of culture fluid. The results are consistent with the view that a family of compounds possessing a similar basal structure are concerned in the cytotoxic activities of *Prymnesium parvum*. The homogeneity of even the most potent of our preparations has not been established. Several of the observed properties of the prymnesins (ichthyotoxicity and haemolytic activity, non-dialysability against water, general solubility features, formation of insoluble inactive complexes with certain alcohols, the capacity to undergo precipitation by magnesium hydroxide and ammonium sulphate) are also exhibited by saponins (see Kofler & Wolkenberg, 1925; Windaus, 1910; Windaus & Weinhold, 1932). But saponin does not require a cofactor.

Though prymnesin in aqueous solution exhibits the behaviour of a colloid, fish immersed in such a solution are promptly sensitized. The rapidity of the action suggests that the immediate target is an exposed organ, probably the gill. Attention has been directed to the fact that the preparations which manifest ichthyotoxicity also have haemolytic activity. Both cation and anion in a salt + prymnesin system affect the toxicity. Moreover, prymnesin causes fish to become susceptible to poisoning by such widely different compounds as calcium chloride and streptomycin sulphate. To account for these observations the hypothesis can be considered that the mode of the action of prymnesin on fish resides in an impairment of the permeability mechanism of the gill.

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Purification of Murray Valley Encephalitis Virus

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SUMMARY

Methods are described for the purification of Murray Valley encephalitis virus from baby mouse brain extract. The method finally chosen involved treatment with protamine, ultracentrifugation, adsorption to and elution from hydroxyl apatite and a second ultracentrifugation. The degrees of recovery of virus infectivity and haemagglutinin were about 15% and 50%, respectively. Measurement of the absorption in the ultraviolet region and examination in the electron microscope suggested that a substantial degree of purification had been achieved. The stability of the infectivity of purified virus was less than that of crude virus. Purified virus yielded infective 'ribonucleic acid' on treatment with phenol. The yield was consistent with the possibility that the potential infective 'ribonucleic acid' in crude virus was contained in infective virus particles.

INTRODUCTION

In 1943 Taylor, Sharp, Beard & Beard showed that purified preparations of eastern equine encephalomyelitis virus contained protein, lipid and ribonucleic acid. This work provided the first indication that the nucleic acid associated with an animal virus might be entirely of the ribose type. Interest in the composition of these viruses has been stimulated by reports that crude preparations of some viruses in this group, upon treatment with phenol, yielded fractions which appeared to consist mainly of ribonucleic acid (RNA) and were infective (Wecker & Schäfer, 1957; Cheng, 1958; Anderson & Ada, 1959). These findings suggest that RNA may carry the genetic information necessary for the production of virus particles which contain lipid as well as nucleoprotein in their structure. The present paper describes a method for purifying Murray Valley encephalitis (MVE) virus from crude extracts of infected baby mouse brains. There was a high recovery rate but the purified virus lost infectivity very rapidly. Nevertheless, it yielded high titre infective 'ribonucleic acid' on treatment with phenol.

METHODS

Saline. An aqueous solution of NaCl (0·15 M).

Bicarbonate cystine saline (*BCS*). A solution of $NaHCO_3$ (0·025 M), cystine (0·85 mM) and NaCl (0·15 M). The cystine was dissolved in saline by boiling, and after cooling the $NaHCO_3$ was added. The solution was filtered through a Seitz sterilizing pad.

Veronal cystine saline (*VCS*). An aqueous solution of sodium barbiturate (0·018 M), HCl (0·009 N), cystine (0·85 mM), NaCl (0·15 M); pH 7·9.

Phosphate buffer. An aqueous solution of K_2HPO_4 (1·8 M) and KH_2PO_4 (0·2 M).

Phosphate saline. An aqueous solution containing NaH_2PO_4 (0·05 M), NaOH (0·044 M), NaCl (0·13 M); pH 7·7.

Phenol. Phenol (A.R.) was distilled *in vacuo*, the distillate saturated with glass-distilled water and stored at 0–4°.

Diethyl ether. This ether was prepared as described previously (Anderson & Ada, 1959).

Normal rabbit serum (*NRS*). Animals in a laboratory colony were bled from the ear vein and serum separated from the clot at 37°. Sera were pooled and filtered through a Seitz sterilizing pad.

Eggs. White Leghorn/Black Orpington cross eggs were incubated at 39° until used and thereafter at 36°.

Source of virus. A strain of Murray Valley encephalitis (MVE) virus, isolated in 1951, was used as 8th mouse passage material. It was inoculated intraperitoneally into mice 5 days old and the brains were harvested when the mice were moribund after a further 4 days.

Preparation of virus extract. Infected baby mouse brains were ground in the appropriate solvent by using a glass mortar fitted with a Teflon pestle which rotated at about 4000 rev./min.

Preparation of infective 'RNA'. The standard procedure described previously (Anderson & Ada, 1959) was slightly modified in that the virus solution was treated twice with phenol, each time for a period of 6 min.

Preparation of normal mouse brain extract. Brains were harvested under chloroform anaesthesia from mice 8 or 9 days old and ground in the desired solvent with the mortar and pestle described above. Grinding was carried out for 3 min. and the fluid clarified by centrifugation (2000 ***g***, 5 min., 0–4°).

Titration of virus and infective 'RNA'. Titration in 12-day chick embryos was done on the chorioallantoic membrane, the membranes being 'dropped' with an inoculum of 0·05 ml. Titration in mice 5–8 days old was by intraperitoneal inoculation of 0·05 ml., or intracerebral injection of 0·01 ml. Infectivity endpoints were determined as 50 % lethal end-points at 4 days in chick embryos; mice were observed for deaths over a period of 12 days.

Haemagglutination. Adult goose red cells were prepared in Alsever's solution and stored for periods up to 4 weeks at 4°. Just before use they were washed three times with saline and made up to 1 % (v/v) suspension in phosphate saline. In the haemagglutination test, haemagglutinin was serially diluted in tenfold falling dilutions in 0·5 ml. volumes of phosphate saline. To each tube was added 0·25 ml. of 1 % goose red cells and the contents mixed. Titrations were read after standing at 22° for 45 min.

'Fluorocarbon' treatment. A proprietory preparation, labelled Freon 113, was used. Equal volumes of infective solution and fluorocarbon were ground at 0–4° in the mortar described earlier. After the treatment the aqueous phase was recovered by centrifugation.

Protamine sulphate. A preparation obtained through the courtesy of the Commonwealth Serum Laboratories, Melbourne.

Hydroxyl apatite. This was prepared according to the technique of Tiselius, Hjertén & Levin (1956). It was stored under phosphate buffer (0·001 M K_2HPO_4, 0·001 M KH_2PO_4; pH 6·8) at 0–4° for periods up to 4 weeks.

Column chromatography. Columns of hydroxyl apatite were prepared about 3 hr. before use. They were pre-washed with about 20 ml. BCS.

Ultracentrifugation. All ultracentrifugation was carried out in a model L Spinco ultracentrifuge fitted with a 40 rotor.

Ultraviolet spectrophotometry. A Uvispek apparatus was used, all readings being made in 0·5 cm. optical cells.

Electron microscopy. A Siemens Type E4 electron microscope was used. The infective sample was treated for 16 hr. at 4° with one half its volume of 3 % (w/v) formaldehyde solution. The sample was then dialysed against distilled water (24 hr.; 4°) and mounted from a high-pressure spray gun on formvar covered grids. The droplets were shadowed with gold-manganin and examined at magnifications of about ×15,000 and at 60 kV with a 50 μ objective aperture.

RESULTS

Preliminary attempts at purification

In early experiments several techniques were tried for their value in the purification of MVE virus. The criteria of usefulness included the degree of purification achieved, the yield of virus and the subsequent stability of the virus. The techniques were applied to solutions of either crude or partially purified virus, and comprised both those which precipitated non-viral material (treatment with protamine, fluorocarbon or ammonium sulphate) and those which achieved a selective concentration of virus particles (sedimentation or column chromatography).

Treatment with protamine. Warren, Weil, Russ & Jeffries (1949) found that protamine precipitated non-viral material from crude preparations of virus but did not decrease the infectivity of the solution. They removed excess protamine with heparin. Protamine behaved similarly with crude preparations of MVE virus. In 13 experiments the change in titre varied from a gain of 1·0 log unit to a loss of 1·0 log unit of infectivity, the mean change being a loss of 0·1 log unit which was not significant. Protamine treatment decreased the u.v. absorption at 260 mμ and 300 mμ by an average of 80 % and 90 %, respectively. The method of purification finally chosen included treatment with protamine but not heparin.

Treatment with fluorocarbon. Gessler, Bender & Parkinson (1956) found that some fluorocarbons when mixed vigorously with crude preparations of certain viruses preferentially removed non-viral material from solution. In the present work, some purification was achieved when MVE virus preparations were briefly treated with fluorocarbon. The best results were obtained when infected fluids, previously treated with protamine, were exposed to fluorocarbon for 5 min. No loss of infectivity occurred, though the % decrease of absorption at 260 mμ and 300 mμ was 26 ± 14 (S.D.) and 45 ± 15 (S.D.), respectively. When virus, which had been purified either by sedimentation in the ultracentrifuge or by ammonium sulphate fractionation as described below, was treated with fluorocarbon, the recovery of infectivity was very erratic.

Ammonium sulphate fractionation. Crude preparations of virus were first treated with protamine, then with fluorocarbon, and finally with ammonium sulphate. In six experiments the mean loss of infectivity due to the ammonium sulphate step was 0·7 log ± 1·2 log unit. In a seventh experiment, dialysis against buffered saline (pH 7·7) at 0–4° for 16 hr. to remove ammonium sulphate, resulted in a further loss of 1·3 log unit. This dialysed material lost another 1·4 log unit of infectivity after one fluorocarbon treatment.

Adsorption to and elution from hydroxyl apatite. The virus present after treatment of crude preparations with protamine could be adsorbed to columns of hydroxyl apatite. In preliminary experiments, 30 ml. protamine-treated extract were adsorbed to a column (3 cm. × 1·5 cm.) of hydroxyl apatite which was then developed with a gradient of phosphate concentration (0·02 M–2·0 M). The virus eluted under these conditions and the infectivity were quantitatively recovered. However the elution profile was very broad and, though marked purification occurred, little concentration was achieved. Later work showed that by using a deposit obtained by centrifugation of the protamine-treated extract, a smaller capacity column could be used and a sharper elution pattern obtained.

Ultracentrifugation. Virus in eluates from hydroxyl apatite columns could be sedimented by ultracentrifugation. In four experiments, high titre eluates were pooled and centrifuged at 90,000 *g* for 30 min. and the rotor then stopped with the brake on. The virus formed a firm pellet at the bottom of the tube. About 95 % of the supernatant fluid was removed by Pasteur pipette and the remainder used for resuspending the virus pellet. In four experiments the average recovery of virus was 100 %, 90 % being in the sediment. When such virus was again centrifuged under the same condition the pellet was not adequately dispersed and the recovery of infectivity was appreciably lower. In the technique finally adopted the conditions of centrifugation were changed in order to overcome this difficulty.

Adopted procedure for purification of MVE virus

The choice of a standard procedure was governed not only by the yield of virus and the degree of purification achieved but also by the lability of the purified virus. Because of this it was desirable that concentration should be accompanied by purification; therefore after protamine treatment the virus was concentrated by sedimentation in the ultracentrifuge. This had the advantage of removing much of the soluble protein so that a smaller column of hydroxyl apatite could be used. The complete procedure took about 6 hr., and all steps were carried out between 0 and 4°. Sterile reagents and containers were used.

Sixty freshly-harvested infected mouse brains were ground in 90 ml. BCS and the solution clarified by centrifugation at 2000 *g* for 5 min. Ten ml. of a warm saline solution containing 360 mg. protamine sulphate was added with stirring to the supernatant fluid (termed 'original extract'). The mixture was shaken mechanically (oscillation/min. = 100; amplitude = 5 cm.) for 30 min. after which a clear supernatant fluid (protamine-treated extract) was obtained by centrifugation (2000 *g*; 10 min.). The protamine-treated extract was centrifuged in a 40-rotor at 93,000 *g* for 15 min., the brake being left off to slow deceleration. The virus was seen as a loose milky concentrate at the bottom of the tube with a small clear pellet to one side. The supernatant fluid was carefully removed, leaving about

0·5 ml. in which the virus was readily resuspended with a Pasteur pipette (termed 'deposit 1').

The pooled deposits (about 5 ml.) were applied to a column (2 cm. × 1 cm.) of hydroxyl apatite and the column washed with 10 ml. BCS. The column was then developed with about 5 ml. portions of BCS to which 2 M phosphate buffer (pH 7·7) had been added to yield final concentrations of 0·025 M, 0·05 M, 0·1 M, 0·2 M, 0·3 M, 0·4 M and 0·5 M phosphate. The flow rate was about 15 ml./hr.

The 0·1 M and 0·2 M eluates were pooled and centrifuged (93,000 *g*, 12 min.). The sedimented virus was then seen as before and was resuspended with a Pasteur pipette in about 0·7 ml. of the supernatant fluid. The products thus obtained were termed 'supernatant 2' and 'deposit 2'.

Evaluation of standard procedure

The standard procedure for purification was applied to nine crude preparations of virus. The various fractions were examined for their % recovery of virus, as estimated by haemagglutination, infectivity, and u.v. absorption. The latter examination also gave some indication of the chemical nature of each fraction.

Recovery of viral haemagglutinin. The original extracts showed haemagglutination in only five of the nine samples; in these five the titres ranged from 2500 to 4000. All this haemagglutinin was recovered between the 0·1, 0·2 and 0·3 M phosphate eluates, and in four of the five cases the haemagglutinin was present almost entirely in the 0·1 and 0·2 M fractions. There was again a quantitative recovery of haemagglutinin in supernatant fluid 2 and in deposit 2, the distribution between these two fractions being in the ratio 1:3·4. In four out of five experiments, the haemagglutinin titre of deposit 2 was between 2×10^5 and 4×10^5.

In those experiments where it was sought, an inhibitor of haemagglutination was found in some fractions. The inhibitor was not titrated in the classical fashion, but some estimate of its amount was obtained by noting the extent of the prozone in a standard titration of haemagglutinin. This prozone extended to the dilutions 1/200, 1/200 and 1/300 in titrations of three original extracts, with haemagglutinin titres of 3000, 3000 and 4000, respectively. A prozone occurred irregularly in the 0·1 M to 0·5 M eluates; in those 0·1 M and 0·2 M eluates where the prozone occurred, it extended to dilutions of 100 to 3000; in the 0·3 M to 0·5 M eluates, to dilutions of 5 to 30. In five experiments the 'titre' of prozone ranged from 10 to 300 in supernatant 2, and in three experiments from 20 to 80 in deposit 2.

It was felt that if sufficient inhibitor were present it might completely obscure haemagglutinin. This was accepted as the explanation of the absence of detectable haemagglutinin in four of the nine original extracts. Four of the nine 0·025 M eluates and five of the 0·05 M eluates also lacked haemagglutinin although the infectivity titres were such that considerable haemagglutinin titres would have been expected. Here too the effect was attributed to an excess of inhibitor.

These experiments throw little light on the nature of the inhibitor(s) of haemagglutinin. In one experiment, normal baby mouse brains were extracted and subjected to the standard fractionation procedure. The ability of the fractions to inhibit viral haemagglutination was tested, the haemagglutinin used being a 0·3 M phosphate eluate prepared on the same day. This had a titre of 4000 and was used at a dilution containing 12 A.D. The original extract was found to have an inhibitor titre of 60; none of the other fractions had any activity.

Recovery of infectivity

In nine experiments the ELD50 of the original extract from infected brain had a mean value of 8·2 log. (range 7·6–8·7). In five experiments the average recovery of infectivity in different fractions was as follows: deposit 1, 71 %; effluent, usually less than 0·001 %; eluate 0·025M, 2 %; eluates 0·1M+0·2M, 70 %; eluates, 0·3M+0·4M+0·5M, 6 %; supernatant 2, 5 %; deposit 2, 15 %. Most of the infectivity was recovered in the 0·1M and 0·2M eluates which were therefore chosen for final centrifugation. The greatest loss was during the final centrifugation.

The ratio of ELD50 to haemagglutinin titre was calculated for certain fractions in three of the experiments where haemagglutinin was present in the original extract. Mean values were: the original extract, $3{\cdot}4 \times 10^4$; deposit 1, $1{\cdot}1 \times 10^4$; the 0·025M eluate, $> 20 \times 10^4$; the 0·1M+0·2M eluates, $1{\cdot}5 \times 10^4$; the 0·3M+0·4M eluates, $0{\cdot}2 \times 10^4$; deposit 2, $0{\cdot}6 \times 10^4$. The ratio for original extract and for combined 0·1M and 0·2M eluates were similar. The high value for the earlier eluate was attributed to the suppression of haemagglutinin by inhibitor; the low value for the later eluates was attributed to dead haemagglutinating virus, a hypothesis which is supported by the low ratios of ELD50 to u.v. absorption values of these eluates. It was first thought that concentrations of phosphate higher than 0·2M might destroy infectivity but the addition of phosphate to 0·2M eluates was found not to decrease the ELD50 value.

U.v. absorption. In Table 1 are recorded the absorption of fractions from normal and from infected brain extracts at 260 mμ and values of the ratio, absorption at 260 mμ: absorption at 280 mμ. In both cases the values quoted were from single experiments. The results from the experiment with infected brain were typical of 5 experiments. As seen in columns 1 and 2 of Table 1, the normal brain absorbed more than the infected brain extract. The protamine extracts of each preparation absorbed to about the same degree. Deposit 1 derived from the infected extract absorbed more than did deposit 1 from the normal extract, because of the presence of virus in the former. The effluent from each column showed similar degrees of absorption. A substantial difference in the elution profile from the infected column compared with the normal column was first apparent at the 0·1M eluate stage. This and later eluates from the normal column contained negligible amounts of material absorbing at 260 mμ, whereas in the infected column these were the very eluates where there was an accumulation of virus. The peak was in the 0·2M eluate consistently in nine experiments. This effect was seen visually in eluates from the infected column, the 0·2M eluate having a greater opacity than the 0·1M and 0·3M eluates. The other fractions were clear, as were all eluates from the normal column.

Of the substances likely to be present in appreciable amounts in the fractions, only three absorbed well at 260 mμ and 270 mμ: namely, proteins and nucleoproteins and small molecular weight purine and pyrimidine derivatives. For each of these, the ratio 260 mμ:280 mμ lay in a typical range; for proteins, it was less than 1; for nucleoproteins, 1 to 2, and for small molecular weight purine and pyrimidine

Table 1. *Ultraviolet absorption values of fractions from normal and from MVE-infected brains, and the infectivity titres of fractions from infected brains*

Values were from one experiment.

Fraction		Volume (ml.)	OD 260 mμ*		OD 260 mμ : OD 280 mμ		Infectivity ($\log_{10}$)
			Normal	Infected	Normal	Infected	
Original extract		90	23·4	14·4	1·26	1·22	8·3
Protamine extract		—	1·80	1·92	1·83	1·84	8·2
Deposit 1		—	2·44	3·24	1·98	1·58	9·4
Column effluent		—	0·74	0·61	2·15	2·4	4·4
Column eluates (M)	0·025	5	0·097	0·109	1·12	1·07	7·0
	0·05	5	0·091	0·113	0·86	0·83	7·5
	0·1	5	0·037	0·238	0·68	1·34	8·2
	0·2	5	0·037	0·579	0·78	1·38	9·2
	0·3	5	0·01	0·212	—	1·37	8·6
	0·4	5	0·01	0·031	—	—	8·2
	0·5	5	0·01	0·01	—	—	—
Supernatant 2		9·3	—	0·132	—	1·18	8·2
Deposit 2		0·7	—	4·42	—	1·56	9·6

* OD = optical density.

derivatives, it was greater than 2. The latter compounds were not deposited under the conditions of the present experiments. As about 70 % of the absorbing material in the 0·1 M and 0·2 M eluates was sedimented and as the remaining 25 % (supernatant 2) had a ratio of 1·18, it seemed that small molecular weight purine and pyrimidine derivatives were virtually absent; the sedimented material with a ratio of 1·56 was therefore largely nucleoprotein. The value of the 260 mμ:280 mμ ratio of the 0·1 M and 0·2 M eluates was higher in the 'infected' column (average 1·36) than in the control column (average 0·73); this difference was mostly due to nucleoprotein.

Properties of purified virus

The properties of deposit 2 were investigated in four ways: u.v. absorption and studies in the electron microscope indicated substantial purity; the purified virus yielded infective 'RNA', but was less stable to heat than was crude virus.

Ultraviolet absorption spectrum. An average absorption curve was calculated from the values for eight preparations of deposit 2. The resulting curve was typical of nucleoproteins. The value of the ratio, 260 mμ:280 mμ, was 1·48 ± 0·06.

Examination in the electron microscope. Plate 1, a micrograph of deposit 2, shows little else in the preparation but virus particles.

Liberation of infective 'RNA' from purified virus preparations. In three experiments infective 'RNA' was made by phenol treatment of original extract and of deposit 2. For this purpose original extract was diluted 1/10 and the final deposit was diluted 1/1000 in normal baby mouse brain extract before phenol treatment; these dilutions were allowed for in the final calculation. The ratio of virus titre to titre of 'RNA' derived from that virus (V:R ratio) was 2·3, 2·4 and 3·6 log (average 2·8) for the three original extracts and 2·0, 3·0 and 3·4 log (average 2·8) for the three corresponding preparations of deposit 2.

Stability studies. The best diluents for crude virus were earlier found to be NRS (10 %, v/v) and mouse brain extract (Anderson & Ada, 1959) in which the virus was comparatively stable. Both crude virus in the form of original extract and purified virus in the form of deposit 2 were diluted 1/100 in NRS (10 %, v/v) and held at 37°. Infective titres were, for crude virus, at 0 min., 8·1 log; at 15 min., 8·1 log; at 60 min., 8·0 log. For purified virus, at 0 min., 9·0 log; at 15 min., 9·8 log; at 60 min., 8·8 log. This increase in titre after 15 min. was found regularly. In one experiment of this type where infectivity titrations were done in triplicate, the average values for purified virus were at 0 min., 9·2 log; at 7½ min., 9·4 log and at 15 min. 9·6 log. End-points in the titration of samples incubated 30 or 60 min. were very close but appeared to indicate a decrease in titre.

Purified virus was less stable in the absence of NRS. It lost infectivity when held at 37° either undiluted or diluted 1/1 in VCS. Typical figures in the latter diluent were, at 0 min., 8·5 log; 30 min., 7·2 log and at 60 min., less than 6·2 log. However, in this diluent, at 20°, virus lost only 0·4 log of infectivity over a period of 60 min.

DISCUSSION

The outstanding impression from the present work was the ease with which semi-purified or purified MVE virus lost infectivity. It was not clear whether this was a reflexion of an intrinsic instability of pure virus or whether it was due to damage caused by the method of purification. The diluent in which pure virus was least unstable was a 1/10 dilution in normal rabbit serum. When diluted in this medium and held at 37° pure virus showed an initial increase in titre and then a slow decay. The early increase of about 0·5 log may have indicated the resurrection of individual virus particles, but was probably due to the disaggregation of clumped virus. The later decay was attributed to instability of virus.

The instability of purified virus made it difficult to assess virus recovery rates during purification. Through the first part of the purification procedure (from original extract to the combined 0·1 M and 0·2 M eluates) there was an average recovery of 70 % of infective virus. Through the second part of the purification procedure (from eluates to final deposit) infectivity was decreased by 73 % but haemagglutinin was not lost at all, and the u.v. absorption results also pointed to a satisfactory recovery of viral material during this phase. It thus seemed that most of the original virus was recovered in deposit 2, and that the final yield was vitiated only by the instability of the infectivity of the purified virus.

The purity of deposit 2 has been estimated both by electron microscopy and by examination in the ultraviolet spectrophotometer. The results of electron microscopy were more easily interpreted, and the pictures left little doubt that virus-like particles comprised most of the material in deposit 2.

From the u.v. absorption pattern it was not possible to determine accurately the degree of purity, but the results were consistent with the belief that deposit 2 was relatively pure virus. First, a comparison of the u.v. absorption profiles of eluates from infected columns and from normal columns suggested that nearly all of the absorbing material in the 0·1 M and 0·2 M phosphate eluates from the infected column was virus, and the small amounts of soluble protein present in these fractions would have been largely eliminated during the final centrifugation. Secondly, the value of the ratio, O.D. 260 mμ:O.D. 280 mμ for 8 preparations of deposit 2 was relatively constant (1·48 ± 0·06). These are the wavelengths of maximum absorption of nucleic acid and protein, respectively, and an alteration in the relative concentrations of these two components between one preparation of deposit 2 and another would have resulted in a substantial change in this ratio. Such a change was not observed and the constancy was taken to indicate that deposit 2 contained mainly one type of particle. Thirdly, the absolute value of this ratio (1·48) was consistent with the major component being nucleoprotein in character.

Some estimate of the degree of purification achieved was obtained from the value of the ratio of haemagglutinin to u.v. absorption. This was 700- to 1100-fold higher for deposit 2 than for the crude brain extract. Although the estimate of the u.v. absorption of the crude extract was unduly high because of light scattering effects, it was concluded that the above ratio meant that a better than 500-fold purification had been achieved.

An obvious next step would have been to determine the number of virus particles per infective unit of purified virus preparation. Many such attempts were made in this laboratory, but all were unsuccessful because of the failure to obtain suspensions consisting mostly of single particles for electron microscopy.

It was found earlier that treatment of crude MVE virus with phenol gave rise to infective 'RNA' (Anderson & Ada, 1959). A prime reason for developing a method of purifying MVE virus was to see whether phenol treatment of pure virus liberated 'RNA' and whether the potential 'RNA' in crude preparations was present only in mature virus particles. The average value of the ratio, infectivity of virus: infectivity of 'RNA' (V:R) was 2·8 for both crude and purified preparations. However, the constancy of this ratio may be misleading. It was indicated above that the infectivity of deposit 2 was lower than was expected from its haemagglutinin titre and optical density. As it was not known whether this loss of infectivity was due to damage to protein, lipid or nucleic acid (see Ada & Anderson, 1959) it was difficult to know the meaning of a value of 2·8 for the V:R ratio. For this reason and because of the batch variation in V:R ratios, it is not possible to state whether all the potential 'RNA' in crude virus preparations is contained in mature virus particles. Nevertheless, it is clear beyond reasonable doubt that purified MVE virus yields infective 'RNA'; the figures were not inconsistent with the idea that the potential infective 'RNA' in crude preparations derived from mature virus.

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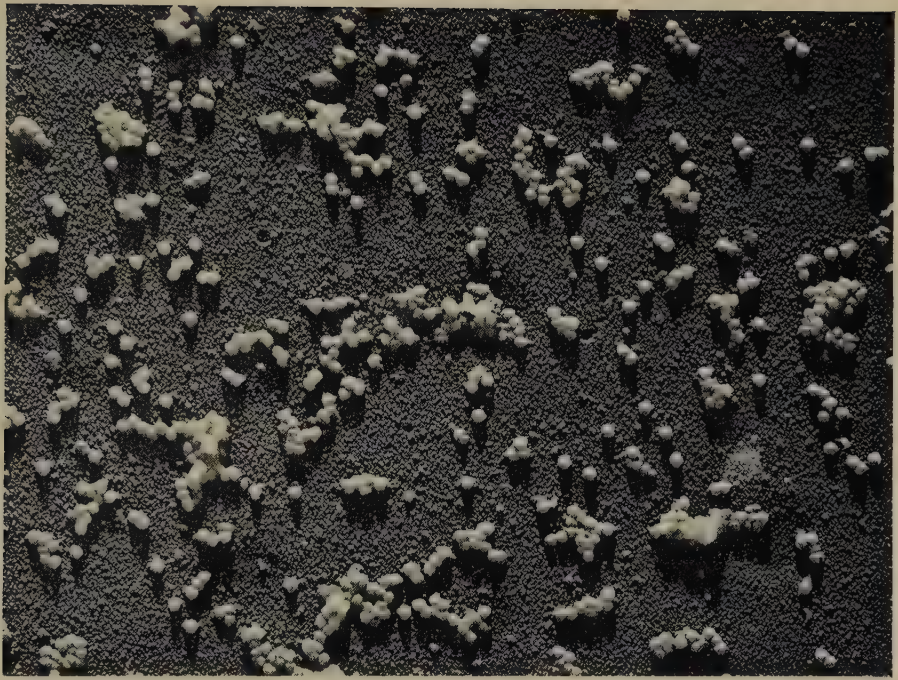
EXPLANATION OF PLATE

Electron micrograph of purified Murray Valley encephalitis virus. Shadowed with gold manganin. Magnification × 40,000.



G. L. ADA, S. G. ANDERSON AND A. ABBOT (*Facing p.* 186)

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A Strain of *Bacillus circulans* Capable of Growing under Highly Alkaline Conditions

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SUMMARY

An organism capable of growing at pH values up to 11·0 appeared, presumably as a contaminant, in the course of ‘training’ experiments designed to produce alkali-resistant strains of *Bacillus cereus* Frankland & Frankland; this organism has now been characterized as *B. circulans* Jordan. Of 26 other strains of *B. circulans* studied, none grew at pH 10·7. The alkali-resistant *B. circulans* strain showed little loss of resistance after many transfers on neutral medium. When it grew in alkaline media it lowered the pH value of these media.

INTRODUCTION

It was reported previously (Kushner & Lisson, 1959) that strains of *Bacillus cereus* Frankland & Frankland, an organism that will not normally grow above pH 9·5, could be ‘trained’ by successive subcultures in media of gradually increasing pH value to grow at pH 10·3. The training process was a long one, requiring more than 50 transfers during more than a month. In four experiments it was not possible to make *B. cereus* grow at pH values higher than 10·3, even when it was transferred daily in media of this pH value for an additional month. In one experiment, however, the culture, which had been slowly trained to grow at pH 10·0 took on, in five transfers, the ability to grow at pH 11·0. Bacteria removed when the culture had acquired the ability to grow at pH 10·0 appeared, microscopically and in colonial form, like the alkali-resistant *B. cereus* described by Kushner & Lisson (1959). Those removed after the culture grew at pH 10·7 and subcultured on neutral or alkaline buffered agar, however, were quite distinct from *B. cereus* in that the rods were thinner than those of *B. cereus* and, on agar, formed small translucent colonies. This organism has now been kept for more than 2 years by transfer on neutral or alkaline buffered nutrient agar. It is not considered likely that it was derived from *B. cereus*, but it is thought to be a contaminant which entered during the training procedure. Because of its ability to grow under highly alkaline conditions, it was further investigated.

METHODS

The conditions of growth for the experiments reported in Fig. 1 were those described earlier (Kushner & Lisson, 1959). Phosphate-buffered nutrient broth (BNB) and phosphate-buffered nutrient agar (BNA), final pH 7·4, were prepared by dissolving dried nutrient broth and dried nutrient agar (Difco) in 0·1 M phosphate buffer (pH 7·5) to give a medium of final pH 7·4. Alkaline media were prepared by the addition of sterile N-NaOH, according to previously determined titration curves. For examining this organism, the methods and media used were similar to those of Smith, Gordon & Clark (1952) and Knight & Proom (1950). Tests were made on BNA (pH 7·4 and 10·7); BNB (pH 7·4 and 10·7); in routine laboratory media (pH 7·0); in media made up in 0·1 M-phosphate buffer (pH 7·5). Incubation was at 37°, except where otherwise stated.

Spore sculpture. The surface configuration of spores was kindly examined for us by Mr D. E. Bradley by the electron microscope and the carbon replica method of Bradley & Williams (1957).

RESULTS

Morphological characters of the highly alkali-resistant organism

The organism conforms in all respects to the description of *Bacillus circulans* given by Smith *et al.* (1952). No difference was found in the morphology on BNA (pH 7·4) and nutrient agar (pH 7·0). On BNA (pH 10·7) at 24 hr. the rods were slightly thinner and longer than on BNA (pH 7·4). The swollen sporangia and oval spores were similar to those formed on BNA (pH 7·4).

Spore sculpture. The sculpture of the spore surface was found by Mr D. E. Bradley by using the electron microscope and carbon replica method of Bradley & Williams (1957) to be smooth.

Growth characters

No difference was obtained from the description of *Bacillus circulans* by Smith *et al.* (1952) apart from the inability to grow anaerobically. No growth was obtained on the surface of nutrient agar and BNA + glucose (1 %, w/v) slopes or streak plates in McIntosh and Fildes jar (aerobic controls positive), and surface growth only in BNA and nutrient agar + glucose (1 %, w/v) shake tubes. The organism appears therefore to be an obligate aerobe. Colonial form and amount of growth appeared similar on BNA (pH 7·4) and BNA (pH 10·7).

Physiological characters

The organism conforms to the description of *Bacillus circulans* by Smith *et al.* (1952) apart from the inability to produce acid from glucose. No growth was obtained on inorganic salts basal medium + sugar up to 7 days at 30° or 37°. Growth but no acid was obtained on nutrient agar slopes with bromcresol purple indicator + glucose, xylose, arabinose, sucrose, mannitol, maltose or raffinose after 7–14 days at 30° or 37°.

Nutrition. The nutritional requirements of this organism were satisfied by a medium containing salts, acid-hydrolysed casein (Knight & Proom, 1950) thiamine and biotin. An ammonia basal medium (Knight & Proom, 1950) did not replace the casein basal medium.

Examination of strains of Bacillus circulans *for alkali resistance*

The above evidence indicated that this organism could be classified as a strain of *Bacillus circulans* Jordan. A collection of 26 strains of *B. circulans* from different sources was therefore examined to assess the occurrence of alkali resistance in this species. Fourteen strains from the Wellcome Research Laboratory collection (Knight & Proom, 1950) and 12 isolated from soil (by M.E.C.) were tested for ability to grow in alkaline media. The cultures were maintained routinely on nutrient agar (pH 7·0) and were subcultured several times in BNB (pH 7·4) at 30° and 37° before testing.

Cultures (24 hr.) in BNB (pH 7·4) at 30° and 37° were inoculated by adding two 4 mm. loopfuls to BNB at pH 7·4 and 10·7. Alkali resistance was indicated by growth in BNB at pH 10·7; results were recorded after growth for 24 hr. at 30° and 37°. None of the 26 strains tested grew in BNB at pH 10·7 in 24 hr.; with the exception of one strain (CN 3378), all grew within 24 hr. in BNB at pH 7·4.

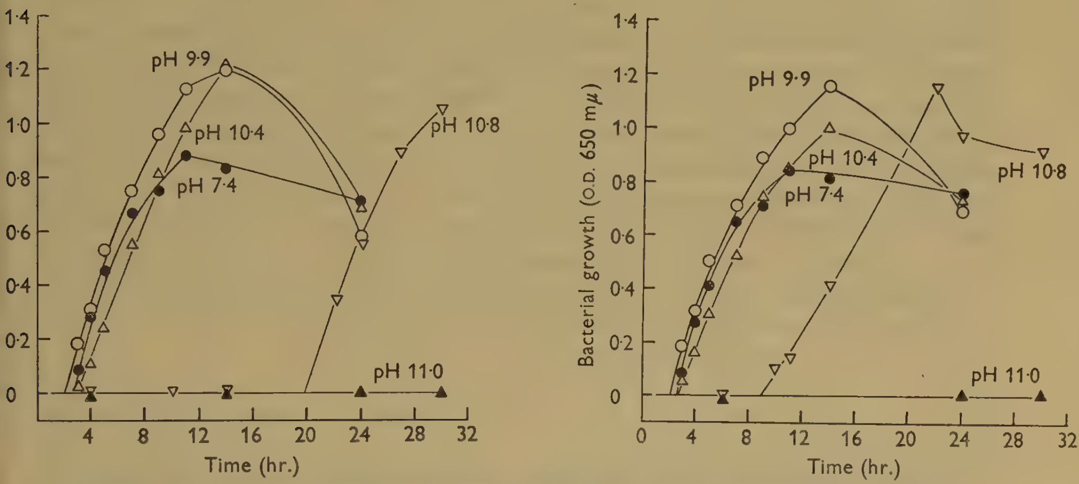

Fig. 1 *a*, *b*. The effect of pH value on growth of alkali-tolerant *Bacillus circulans*. *a*, Bacteria maintained for 1 year at pH 7·4; *b*, bacteria maintained for 1 year at pH 10·7. Bacteria were grown for 20 hr. on BNA of pH 7·4 (*a*) or 10·7 (*b*), resuspended in BNB (pH 7·4) and an inoculum equiv. 0·025 mg. dry wt. bacteria in 0·3–0·5 ml. broth added to tubes containing 10 ml. BNB of the pH values shown. Incubation, shaking, and measurement of optical density were as described by Kushner & Lisson (1959).

Growth at different pH *values and the retention of alkali tolerance*

The effect of the pH value of the medium on the growth of this organism in BNB is shown in Fig. 1. Comparison was made between bacteria which had been maintained for 1 year on BNA (pH 7·4) with transfers at 2-week intervals (Fig. 1*a*) and bacteria which had been similarly maintained at pH 10·7 (Fig. 1*b*). Both cultures grew after a slightly shorter lag period at pH 9·9 than at pH 7·4. In the highly alkaline media in which growth occurred, cultures reached a higher density than they did at pH 7·4. A 1-year long transfer on BNA of pH 7·4 had caused little loss of alkali resistance, the only difference between the two cultures appearing at pH 10·8, where the bacteria maintained at pH 7·4 grew after a lag period 11 hr. longer than did those maintained at pH 10·7. When first isolated, these bacteria grew in shaken cultures of pH 11·0. Two years later, at the time of the experiment shown in Fig. 1, they still grew on BNA at pH 11·0 but did not grow in shaken BNB of this pH value. In this, they resemble alkali-resistant *B. cereus* (Kushner & Lisson, 1959), which grew at a slightly higher pH value on agar than in liquid medium.

In the late stages of incubation with shaking, there was a decrease in the optical density of all cultures. No clumping of the bacteria was observed, and the culture became viscous, indicating that lysis was probably taking place. As with alkali-resistant *Bacillus cereus*, the growth of the present organism in alkaline media lowered the pH value. After incubation for 30 hr. the pH value of the culture which was originally at 7·4 had increased to 7·8, and the pH values of all other cultures had decreased to 8·8–9·0.

DISCUSSION

The swollen sporangia and oval spores produced by this alkali-resistant organism indicate that it is a *Bacillus* sp. of morphological group 2 (Smith *et al.* 1952). The results of the physiological tests classify the organism as a strain of *Bacillus circulans*. Inability to produce acid from glucose and to grow anaerobically is, however, not typical of *B. circulans*, although atypical reactions of this kind are not uncommon with strains of this species-complex. The smooth surface sculpture of the spores is similar to that found in some strains of *B. circulans*, although other strains of this species may show longitudinal parallel ribs or reticulation (Bradley & Franklin, 1958). The nutritional requirements of this strain are also consistent with those found by Knight & Proom (1950) for some strains of *B. circulans* and for *Bacillus* species in general. Since alkali resistance was not found in 25 other strains of *B. circulans* the high resistance shown by this particular strain is, thus far, unique.

The alkali-resistant *Bacillus circulans* strain has been sent to the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen.

We are indebted to Mr D. E. Bradley (A.E.I. Research Laboratories, Aldermaston Court, Berkshire) for the examination of the spore sculpture by the carbon replica method, and to Professor B. C. J. G. Knight for advice during part of this investigation. Mr T. A. Lisson provided valuable technical assistance during the early stages of this work. This paper is contribution no. 653, Forest Biology Division, Research Branch, Department of Agriculture, Ottawa, Canada.

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The Effect of Oxygen on Freeze-dried *Escherichia coli*

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SUMMARY

When *Escherichia coli* organisms were suspended in distilled water and freeze-dried the maximum loss of viability did not occur during the drying process proper, but during the time of contact of the dried organisms with air between the primary and the secondary drying periods. By substituting other gases for air at this stage, it was proven that oxygen was the active agent involved. The dried organisms which were exposed to different pressures of air and oxygen at different temperatures proved to be extremely sensitive to traces of oxygen, even at very low temperatures. The implications of this oxygen effect in connexion with existing freeze-drying procedures, as well as some preliminary kinetic experiments concerning the shape of the survival curve, are discussed.

INTRODUCTION

High viabilities of bacterial suspensions after freeze-drying can usually be obtained only when the organisms are suspended in quite complex media during lyophilization. A survey of the literature (see, for example, Harris, 1954) shows that the discovery of these media has usually beeen a 'hit and miss' affair, and only in exceptional cases was an attempt made to rationalize the success of a certain medium (Fry & Greaves, 1951; Naylor & Smith, 1946). The lethal effect of freezing and drying on bacteria is indeed only very poorly understood, if at all. Studies of the nature of the damage caused by freezing different micro-organisms (Mazur, 1959; this article gives references to most of the current work) have failed to prove that freezing causes the principal or even any important damage during the process of lyophilization. Organisms which withstand freezing well may be killed by lyophilization when not protected by a suitable medium. In addition to freezing, there appeared therefore to exist one or more lethal stages in the drying process itself. An attempt to determine the viability of the bacteria, not only after the completion of the freeze-drying process, but also during its different stages, has apparently been made only once (Fry & Greaves, 1951) and then, as will be shown further below, not under the most suitable conditions.

Most of the work dealing with lyophilization so far has been concerned with finding a medium in which the highest viability of the freeze-dried bacteria could be obtained. Under these circumstances it would be difficult to detect a phase of maximum killing, even if it existed, since when the overall viability of the freeze-dried bacteria remained comparatively high during the whole process, the statistical fluctuations in the viability determinations would be large enough to obscure a decrease in the viability during any one of the stages of freeze-drying. If one carries out the lyophilization of bacteria, which are resistant to freezing, under conditions under which high mortality is to be expected in the drying process, it should be possible to find out whether the mortality in this process is gradual, or whether it is confined to a single defined stage of the drying. As it has long been known (Fry, 1954; and confirmed by us) that freeze-drying of *Escherichia coli* in distilled water yields very poor results, we used this method to get high mortality in the lyophilization process. Moreover, freeze-drying from distilled water would leave the dried bacteria free from the components of the medium, and the results would be likely to be more generally valid. As the test organism has to be washed with, and suspended in, distilled water, the bacterium should be resistant to osmotic shock; *E. coli* has this property.

METHODS

Organism. A locally isolated strain of *Escherichia coli*, E/65, was used, unless otherwise indicated.

Culture methods. Organisms grown on freshly prepared nutrient agar slopes for 24 hr. at 37°, were washed off with, and re-suspended in, sterile nutrient broth to a concentration of approximately 10^7 organisms/ml. This suspension was used to inoculate Roux bottles containing Difco nutrient broth solidified with about 2·5% (w/v) agar. After incubation for 20 hr. at 37°, the organisms were washed off into distilled water, sedimented at 20,000 *g*, washed well with distilled water and then re-suspended in the same medium. This procedure was repeated. Finally, the organisms were resuspended in distilled water to a concentration of $1{\cdot}5\text{–}2{\cdot}0 \times 10^{11}$ organisms/ml. This suspension, from a sample of which a viable count was made, was then used for the lyophilization experiments.

Viable counts. Counts were performed by diluting a sample in gelatin buffer (0·5%, w/v, gelatine in M/15 phosphate buffer, pH 6·8). Portions (0·5 ml.) of the appropriate dilutions were mixed with melted nutrient agar at 44° in Petri dishes. At least three dishes were used at each dilution. After the agar had solidified, the dishes were inverted and opened on wire screens in a forced-air incubator for 15 min. This procedure completely prevented spreading of colonies on the agar surface as well as between the agar and the bottom of the dish. The dishes were then closed and incubated for 48 hr. at 37°, and the colonies counted. Dilutions were selected so as to obtain between 50 and 500 colonies/dish.

Lyophilization

Apparatus. The apparatus used was of the simple manifold type. The ampoules were connected through high-vacuum rubber tubing to the manifold, the diameter of which was large enough to offer only negligible resistance to vapour flow. The condenser consisted of a stainless steel Dewar-type flask and was cooled by a solid CO_2 + ethanol mixture. By a single stage rotary oil pump, a pressure of less than 10μ Hg was produced; the pump was protected from water vapour by a phosphorus pentoxide trap. The pressure in the system was measured by a Pirani gauge connected to the system through a water vapour trap. Air which was bled into the system after lyophilization, was dried by passing it through sulphuric acid, soda lime and through a small-bore long U-tube immersed in a solid CO_2 + ethanol mixture.

The secondary drying system, which was used in part of our experiments, consisted of a long large-bore horizontal cylinder which contained phosphorus pentoxide on stainless steel trays. Ampoules were connected to this system through manifolds. This system was evacuated by a double stage rotary oil pump to a pressure less than 0·5μ Hg.

Lyophilization procedure. The bacterial suspensions were distributed in 1 ml. amounts into (neutral) glass ampoules (outside diameter 15 mm.; length 50 mm.). The contents of the ampoules were quick-frozen in a solid CO_2 + ethanol mixture and stored overnight at a temperature not exceeding −70°. This storage did not affect the viability of the suspensions. The ampoules to be lyophilized were connected to the manifold while still immersed in the freezing mixture until a vacuum of at least 50μ Hg was reached in the drying system. When this pressure had been obtained, the freezing mixture was removed and drying continued, the heat of sublimation being supplied by the surrounding atmosphere. Primary drying, as judged by the disappearance of moisture from the outside of the ampoule, was completed after 20–30 min. By that time the contents of the ampoule had been converted into a fluffy powder. The disappearance of moisture from the outside of the ampoule as criterion of dryness is only a very crude indication and depends among many other factors on the outside temperature and dew point. The drying was, therefore, continued for at least another 60–90 min. When secondary drying was required, dry air was bled into the system, then the ampoules were disconnected from the manifold, their necks drawn out in a small flame, and the ampoules were connected as soon as possible to the secondary drying system. After completion of the secondary drying the ampoules were sealed under vacuum.

For viable counts, the contents of the ampoules were reconstituted by adding gelatin + buffer immediately after opening the sealed ampoules, and the counts performed as soon as possible (never later than 20–30 min. after reconstitution, the material being kept meanwhile in the refrigerator).

RESULTS

To ascertain the point at which the bacteria were killed in the freeze-drying, viability curves were taken at every stage of the freeze-drying of a suspension of *Escherichia coli* in distilled water (freezing, primary drying, admitting dry air into the ampoules, drawing out and constricting the necks of the ampoules, secondary drying, sealing in vacuum). The counts were performed at intervals as short as technically feasible. The contents of three ampoules were reconstituted and their volume adjusted to that of the original sample. Table 1 represents the protocol of a typical experiment; the results are summarized in Fig. 1.

No significant change in viability occurred after freezing and storing in the frozen state, and only a small and gradual drop was found during the primary drying; at the end of that period 50% of the original population was still viable, although lyophilization was carried out in distilled water. On the other hand, a sudden drop in viability occurred between the end of the primary and the beginning of the secondary drying. During the secondary drying itself, there occurred only a small and gradual loss of viability.

Table 1. *Viability of* Escherichia coli *during lyophilization from distilled water*

Time from start of drying (min.)	Manipulation	Viable organisms/ml.				Relative viability (%)
		Sample 1	Sample 2	Sample 3	Average	
—	Before freezing	$1\cdot2\times10^{11}$	$1\cdot2\times10^{11}$	$1\cdot3\times10^{11}$	$1\cdot3\times10^{11}$	100
0	After 20 hr. at −70° drying starts	$1\cdot2\times10^{11}$	$1\cdot1\times10^{11}$	$1\cdot2\times10^{11}$	$1\cdot2\times10^{11}$	92
15	—	$1\cdot2\times10^{11}$	$1\cdot2\times10^{11}$	$1\cdot1\times10^{11}$	$1\cdot2\times10^{11}$	91
30	—	$1\cdot1\times10^{11}$	$1\cdot0\times10^{11}$	$9\cdot8\times10^{10}$	$1\cdot0\times10^{11}$	78
45	—	$9\cdot8\times10^{10}$	$9\cdot4\times10^{10}$	$1\cdot1\times10^{11}$	$9\cdot8\times10^{10}$	77
75	—	$7\cdot9\times10^{10}$	$9\cdot0\times10^{10}$	$8\cdot7\times10^{10}$	$8\cdot5\times10^{10}$	67
110	End of drying; air admitted	$7\cdot8\times10^{10}$	$8\cdot1\times10^{10}$	$5\cdot0\times10^{10}$	$6\cdot3\times10^{10}$	50
160	Secondary drying starts	$9\cdot7\times10^{9}$	$6\cdot4\times10^{9}$	$6\cdot4\times10^{9}$	$7\cdot8\times10^{9}$	6·1
215	—	$5\cdot8\times10^{9}$	$4\cdot2\times10^{9}$	$3\cdot8\times10^{9}$	$4\cdot6\times10^{9}$	3·6
355	End of secondary drying	$5\cdot8\times10^{9}$	$6\cdot1\times10^{9}$	$3\cdot7\times10^{9}$	$5\cdot2\times10^{9}$	4·1

The high mortality between primary and secondary drying may be ascribed to either of the following causes: (1) the change of pressure experienced by the organisms, when they were brought from vacuum to atmospheric pressure; (2) the effect of air *per se*. The rise in temperature of the dry powder during the constriction of the neck of the ampoule had no influence on the process.

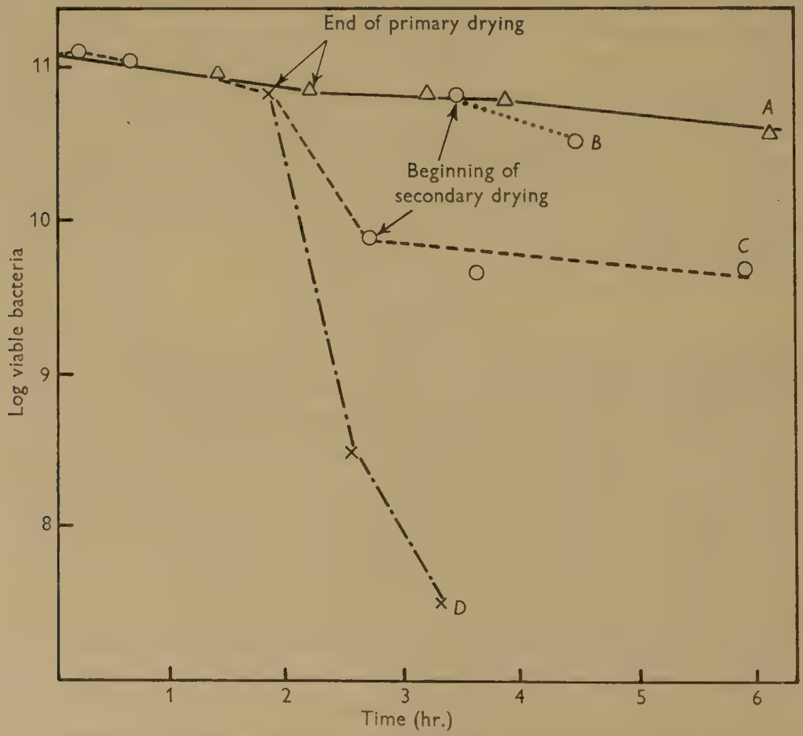

Fig. 1. Viability of *Escherichia coli* suspended in distilled water during the lyophilization procedure. The organisms were exposed to different atmospheres between the primary and the secondary drying: *A*, in vacuum; *B* in hydrogen; *C* in air; *D* in oxygen.

Organisms dried from distilled water were exposed to different gases at atmospheric pressure and at 28°. As controls, organisms were kept under vacuum for the same time and temperature. In view of the above results, the secondary drying was omitted. The results for hydrogen, air, oxygen, and vacuum for *Escherichia coli* E/65 are given in Fig. 1, and for oxygen, air and vacuum for *E. coli* B/r in Fig. 2. The results for these two organisms were similar, except that the B/r strain appeared to be somewhat less sensitive. The viability counts for nitrogen were puzzling at first: the mortality was quite high as compared to hydrogen, although much lower than for air. Experiments showed that the purification of commercial nitrogen by alkaline pyrogallol (5%, w/v, pyrogallol in a 50%, w/v, aqueous solution of potassium hydroxide) was not efficient. When Fieser's solution (Fieser, 1924; 16%, w/v, $Na_2S_2O_4$, 4%, w/v, sodium β-anthraquinone sulphonate in a 14%, w/v, aqueous solution of sodium hydroxide) was used, better results were obtained. They are summarized in Fig. 3.

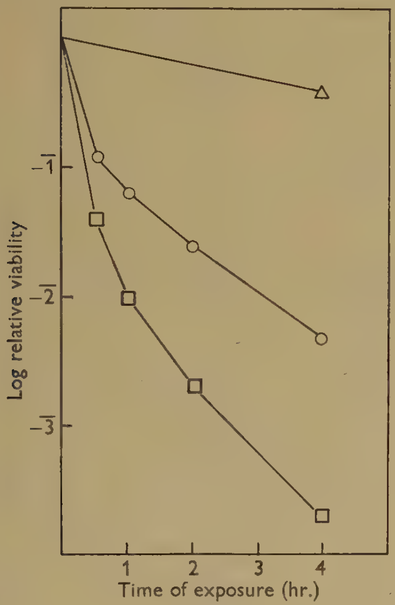

Fig. 2

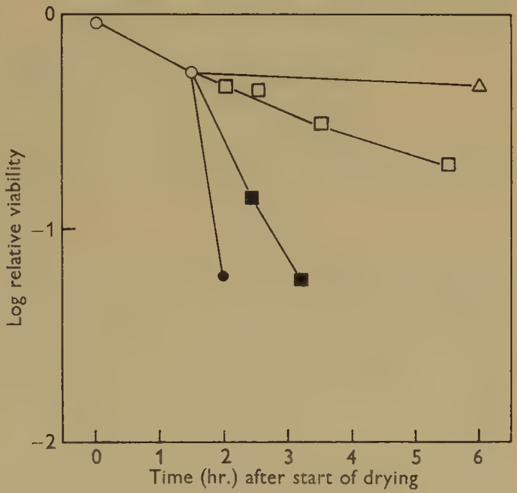

Fig. 3

Fig. 2. Exposure of *Escherichia coli* B/r, dried from distilled water to vacuum, air or oxygen at 28°. △, vacuum; ○, air; □, oxygen.

Fig. 3. Viability of *Escherichia coli* dried from distilled water and exposed to nitrogen 'purified' from oxygen by different methods. △, vacuum; ●, air; □, nitrogen purified by Fieser's solution; ■, nitrogen purified by alkaline pyrogallol.

As the efficiency of the removal of oxygen from the nitrogen increased the viability of the dried suspensions exposed to nitrogen increased. On the other hand, even Fieser's solution did not seem to absorb all traces of oxygen under the conditions used by us. As will be shown presently, the suspensions dried from distilled water are extremely sensitive even to minute traces of oxygen.

Effect of changes in the pressure of oxygen and in the temperature of exposure

Figure 4 shows that the viability of the dried suspension exposed to oxygen for 3 hr. at room temperature depended on the oxygen pressure. The extreme sensitivity of the suspensions to quite low oxygen pressures was pronounced; about 95% of these organisms were killed in 3 hr. by oxygen at a pressure as low as 10 mm. Hg. (In these experiments part of the pressure range was covered by exposure to air, part by exposure to oxygen. Contrary to expectations, in the overlapping region of these two curves the results were not identical; it is not clear at present what caused the discrepancy.)

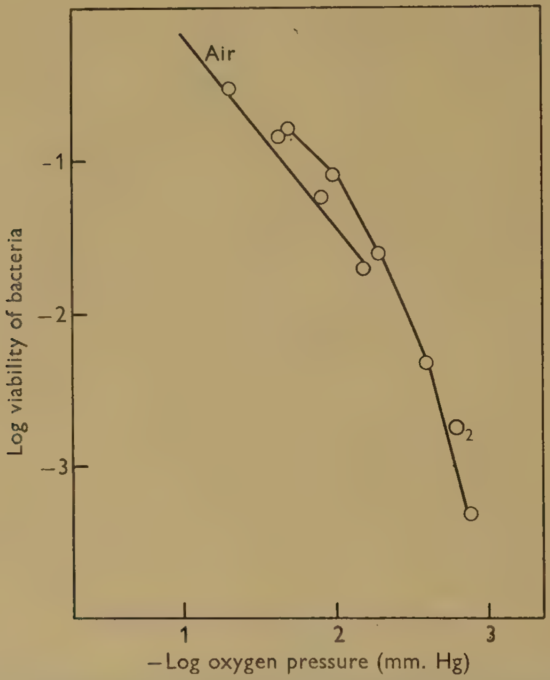

Fig. 4. Viability of *Escherichia coli* dried from distilled water and exposed to air and oxygen at different pressures for three hours at room temperature.

Figure 5 describes the viability of the dried suspensions when exposed to air at atmospheric pressure for 3 hr., but at different temperatures. Although the reaction between oxygen and the dried suspension was highly temperature-dependent, the mortality of the dried suspensions exposed to oxygen was easily measurable even at temperature as low as –30°. When the exposure was prolonged beyond 3 hr., killing was evident at even lower temperatures.

Shape of the survival curves

As seen in Fig. 2, the plot of the logarithm of the survival in dried suspensions exposed to air or oxygen against time of exposure yielded a curve which is concave upwards. This may have been due to a consumption of oxygen and corresponding

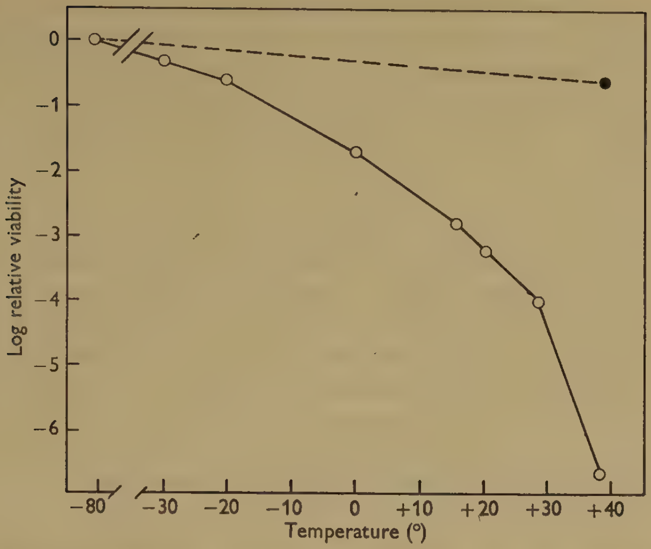

Fig. 5. Viability of *Escherichia coli* dried from distilled water and exposed to air at atmospheric pressure for three hours at different temperatures. ●, control in vacuum; ○, exposed to air.

fall in its pressure, which in turn would cause a slowing down of the reaction rate. That this is not the case is shown in Table 2, in which two conditions are compared: in one case the organisms were exposed to the normal volume of oxygen, while in the other a volume about twenty times as large was used. The results were not significantly different. In another experiment described in Table 3 different amounts of organisms were exposed to the same volume of oxygen. The results show that in

Table 2. *Viability of* Escherichia coli *freeze-dried from distilled water and exposed to different volumes of air at atmospheric pressure at* 28°.

Original density of suspension $1{\cdot}2\times10^{11}$/ml. Each figure represents the average of three samples.

Volume of air (ml.)	Period of exposure to air (min.) 30	120	240
	Viability (%)		
10	8·0	0·7	0·05
200	5·8	0·4	0·06

Table 3. *Viability of* Escherichia coli *freeze-dried from distilled water at different concentrations of suspension, and exposed to air at atmospheric pressure at* 28°.

Original concentration of organisms	Period of exposure to air (min.) 60	120	240
	Viability (%)		
$1{\cdot}5\times10^{11}$/ml.	1·1	0·3	0·03
$1{\cdot}5\times10^{10}$/ml.	1·4	0·5	0·03

this case the relative inactivation of the suspensions was independent of the original concentration of organisms. We see, therefore, that on the one hand the organisms were inactivated independently of each other, and on the other that the concentration of the inactivating agent was not rate-limiting under the conditions of our experiment. One would, therefore, expect an exponential survival curve. This point is discussed below.

DISCUSSION

The fact that oxygen is deleterious to lyophilized bacteria has been recognized for a long time (Rogers, 1914); for this reason freeze-dried substances are generally stored under vacuum or in inert gases. In some instances authors have tried to decrease the mortality in lyophilized material by adding anti-oxidants to the suspending medium (Naylor & Smith, 1946); however, it is difficult to see how anti-oxidants are able to counteract the oxidation by traces of air in the dry state.

Experiments carried out to determine the effect of different gases on freeze-dried bacteria (Naylor & Smith, 1946) demonstrated the lethal effect of air. This effect was a slow one and could be measured only after extended periods of storage; a 90% decrease in viable count was found for *Serratia marcescens* after storage in air for 48 days, as compared with an insignificant change when stored under vacuum. Interestingly enough, a decrease in viable count of 70–75% was found when these organisms were stored under tank nitrogen, or under nitrogen 'purified' (from oxygen) by alkaline pyrogallol. Similarly, unsatisfactory results with dry nitrogen were reported by Collier (1954). These results are not surprising in the light of the work described here.

Much more attention has usually been paid to the exclusion of moisture from the freeze-dried material than to the prevention of contact with air. Fry & Greaves (1951) sealed their ampoules under vacuum, rather to exclude unknown amounts of moisture than to exclude traces of air. Swift (1937), Stillman (1941), and Morton & Pulaski (1938) used dry air as storage atmosphere for their material, but this practice is no longer accepted (Fry, 1954). Meryman (1959) even suggested drying frozen material by passing dry air over it, instead of using the conventional vacuum. It seems, therefore, that the effect of oxygen on dried cells has been generally underestimated. In our work two facts have come to light. (1) It is possible to obtain a high survival in suspensions freeze-dried from distilled water, i.e. without any protective medium, when these suspensions are carefully protected from traces of oxygen. This is true at least in the case of *Escherichia coli*, which is resistant to hypotonic shock and to freezing in distilled water. (2) The failure reported by other workers in the lyophilization of suspensions from distilled water is due to the extreme sensitivity of the organisms to air. In the process of primary drying the neck of the ampoule should have a bore as large as possible. Since it is very difficult to seal such a wide neck under vacuum, the usual procedure is to let dry air into the system after the primary drying and to constrict the neck of the ampoule before the secondary drying. It is seen now that under these conditions drying of organisms from distilled water would not be successful. Substituting commercial nitrogen (even when 'purified' by conventional methods) for air between the primary and secondary drying, would not help very much, unless it is freed from oxygen by highly efficient reagents. Naylor's experiments (Naylor & Smith, 1946), mentioned above, which demonstrated only a much smaller effect of air on freeze-dried suspensions, were performed on bacteria which were embedded in the drying medium devised by these workers. This would suggest that their medium contained a substance protecting the dried organisms from oxygen. This has indeed been proven to be the case (Lion & Bergmann, unpublished).

Although careful exclusion of air prevented the death of most of the *Escherichia coli* organisms freeze-dried from distilled water, yet 50% were killed during the primary drying. This might be due to the traces of air diffusing through imperfect connexions of a vacuum system. Some air is also liberated during evacuation from the inner walls of the system, and furthermore, the small bubbles of air originally dissolved in water become trapped in the ice when the suspension is frozen and are liberated during freeze-drying when the ice evaporates. Although these traces of air may account fully for the loss of viability during the drying process, other causes of mortality are by no means excluded and, furthermore, the death of organisms during storage of freeze-dried material may occur even in the complete absence of oxygen. Complete absence of even traces of oxygen is extremely difficult to achieve, and these traces may have a long time in which to react with the organisms.

Some remarks about the shape of the survival curve depicted in Fig. 2 may be in order; its form is rather difficult to interpret kinetically. In our experiments oxygen was in excess. On the other hand, the relative inactivation rate seemed to be independent of the initial density of the suspension of organisms; thus no interaction between the organisms seemed to occur. Under these conditions, a first-order inactivation curve would be expected for a homogeneous population. As this was not the case, some heterogeneity must be present. The hypothesis that part of the population is genetically more resistant to oxygen is highly unlikely, since no mutant more resistant to freeze-drying (which nearly always includes such an oxygen effect) than the original population has ever been isolated. A metabolic or physiological heterogeneity cannot be excluded, although in our case it was not very probable, since the organisms were harvested long after the completion of the log phase and very carefully washed with distilled water before use. In general, our curve resembles very much the inactivation curve of poliomyelitis virus by formaldehyde. An analogous model to that proposed by Gard (1957) for this virus might be formulated with some justification. We believe, however, that more detailed kinetic studies should first be performed. The similarity between the inactivation curves of dried bacteria by oxygen and of virus by formaldehyde leads us to expect that the lethal effect of oxygen on unprotected freeze-dried bacteria may be utilized to prepare vaccines from killed bacteria. This method is now under investigation.

This paper forms part of a thesis submitted to the Hebrew University (by M.B.L.) in partial fulfilment of the requirements for the degree of Ph.D.

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Inter-species Change in Thiobacilli

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SUMMARY

Inter-species conversion has been observed in thiobacilli, starting from isolates of single bacilli.

INTRODUCTION

Since the early studies on the thiobacilli by Nathansohn (1902) and Beijerinck (1904) work has been directed mainly to the elucidation of their metabolic pathways and their classification. During an investigation into the organisms responsible for the oxidation of sulphur compounds in gas works effluents, an attempt was made to classify the bacteria concerned. It soon became apparent that the classification given by Parker (1957) was inadequate, largely because most of the species had been studied by different investigators using different media and criteria. Several species were therefore obtained from the National Collection of Industrial Bacteria (NCIB) for comparative purposes. A note on the anomalous behaviour of *Thiobacillus thioparus* NCIB 8370 was published by Townshend & White (1960); this paper is concerned with the reason for this discrepancy.

METHODS

Organisms. Two of the organisms used were very similar, and as they closely resemble *Thiobacillus neapolitanus*, they will be referred to as 'neapolitanus type'. These organisms were: 'G' which was isolated from a laboratory activated sludge unit treating thiocyanate, and no. 5 which was given by Miss Adams of the National Chemical Laboratory. The other culture used, NCIB 8370, will be referred to in the paper as 8370.

Culture media. Oxoid Nutrient Agar was used for the nutrient media, and Oxoid Ion Agar no. 2 for the inorganic media, both obtained from Oxo Ltd. London. The various inorganic media used are given in Table 1. Medium S8 was used for anaerobic culture in a McIntosh and Fildes jar.

Stock cultures. These were maintained on S2 agar slopes at 10° and subcultured every 3 weeks. All incubations were at 30°.

Single organism isolations. These were carried out on the surface of an agar block, using a modification of the technique described by one of us (Johnstone, 1953) with phase contrast illumination. Portions of the block, each carrying a single organism, were transferred to 10 ml. amounts of the S2 medium in 50 ml. conical flasks.

Table 1. *Composition of inorganic culture media (g./l. distilled water)*

	Media				
Compound	S1	S2	S5	S7	S8*
$Na_2S_2O_35H_2O$	10·0	10·0	10·0	—	10·0
NH_4CNS	—	—	—	0·2	—
$Na_2HPO_412H_2O$	2·0	4·0	—	3·0	3·0
KH_2PO_4	—	4·0	2·0	1·8	1·8
$MgSO_27H_2O$	0·1	0·1	0·1	0·1	0·1
$(NH_4)_2SO_4$	0·1	0·1	0·1	0·1	0·1
$CaCl_2H_2O$	0·1	0·1	0·1	0·1	0·1
KNO_3	—	—	—	1·0	5·0
Na_2CO_3	0·2	0·2	0·2	0·2	0·2
$FeCl_36H_2O$	0·02	0·02	0·02	0·02	0·02
$MnSO_44H_2O$	0·02	0·02	0·02	0·02	0·02
pH	7·8	6·7	5·2	6·7	6·7

* Anaerobic.

RESULTS

Experiments with organism 'G'

In the preliminary isolation of organisms from the activated sludge unit, a pure culture was obtained by repeated single colony isolation; it closely resembled *Thiobacillus neapolitanus* but differed in showing a variable ability to decrease the pH value. Organism G was a strictly aerobic autotroph, which decomposed only thiosulphate media, and gave rise to a 'w' colony type (Table 2). Stock cultures showed colony variants within 2 months. These consisted of two colony types, one a round colourless colony not readily distinguishable from 'G' except that no sulphur was deposited, and a very thin flat colony with a crenate edge. One or both of these colonies appeared each time 'G' was re-isolated, but no other colony types were observed. Both these organisms grew on nutrient agar, the round colony type gave rise to a round greyish white colony, and the crenate type to a round opaque grey colony, around which the medium became brown after 7 days. Pure cultures of these organisms grew in thiosulphate liquid medium, but whereas the round colony type used the thiosulphate slowly, the breakdown of thiosulphate by the crenate organism was negligible. Attempts to obtain these variants from the laboratory atmosphere by exposing both liquid and solid media were unsuccessful. Although a mould grew in the liquid medium, no thiobacilli or the colony types described above were ever found.

At this time a culture of *Thiobacillus thioparus* NCIB 8370, was obtained, which gave unexpected results (Table 2), and an attempt was made to obtain a pure culture of *T. thioparus* from it, by means of single cell technique.

First series of isolation experiments

In the first series of isolations 8370 was grown on S2 liquid medium and the bacteria isolated during the log phase. The isolates were transferred to the S2 medium, and subcultured thence to various test media (Table 2). As may be seen from Table 2, seven of the twelve isolates grew, five of those within 5 days and a further two (I.7 and I.12) after 2 weeks. Four types of bacteria were dis-

Table 2. *Character of the isolates from NCIB* 8370 (*series* 1)

Isolate no.	Loss of sulphur compound in liquid media				Growth on agar plates (2 % agar)					Colony* type
	S1	S2	S5	S7†	S1	S2	S5	S8‡	Nutrient	
I.4	+	+	+	+	+	−	+	−	−	w
I.6	−	−	−	−	+	+	+	+	+	r
I.7	+	+	+	+	+	−	+	+	−	w
I.8	−	−	−	−	+	+	+	+	+	r+c
I.9	+	+	+	+	+	+	+	+	+	r+c+w
I.10	+	+	+	+	+	+	+	+	+	r+w
I.12	+	+	+	+	+	−	+	+	−	w
Original 8370	+	+	−	−	+	+	−	+	+	w

* Colony types: w = round colony which deposited sulphur; r = round colony which did not deposit sulphur; c = crenate-edged colony which did not deposit sulphur.
† Thiocyanate. ‡ Anaerobic.

tinguishable by the test media used, I.4 ('w' colony type, strict aerobe) I.6 ('r' colony type, facultative anaerobe) 1.7 ('w' colony type facultative anaerobe), crenate organism ('c' colony type, strict aerobe). The distribution of the three colony types on various solid media (Table 3) shows clear differences between the four organisms. I.4 and I.7 were strictly autotrophic thiobacilli with similar colonial appearance, which decomposed thiosulphate and thiocyanate, but which differed in I.7's ability to grow anaerobically. I.6 was a heterotroph and a facultative anaerobe, which decomposed thiosulphate slowly. The crenate colony type was strictly aerobic and a heterotroph.

Table 3. *Colony types appearing on the agar plates* (*Series* 1)

	Media				
	Colony type				
Isolate no.	S1	S2	S5	S8‡	Nutrient
I.4	w	—	w	—	—
I.6	r	r	r	r	r
I.7	w	—	w	w	—
I.8	r+c	r+c	r+c	r	r+c
I.9	r+c+w	r+c	r+c+w	r	r+c
I.10	r+w	r	r+w	r	r
I.12	w	—	w	w	—

Colony types: As in Table 2 above. ‡ Anaerobic.

The most interesting aspect of this work was that in three cases a single bacterium had given rise to more than one type of organism. In addition, the two organisms which did not deposit sulphur on the plates were very similar to the variants found previously with 'G'. At this time the stock cultures of 8370 and 'G' were again tested, and 8370 had acquired the ability to decompose thiocyanate, thus paralleling the changes found in the isolates. 'G' also utilized thiocyanate, and the two stock cultures now appeared to be identical, each growing on all the test media and exhibiting the three colony types.

Table 4. *Characteristics of the isolates from* 8370 *and organism* no. 5 (*series* 2)

Isolate no. 8370	Loss of sulphur compound in liquid media				Growth on agar plates					Colony* type
	S1	S2	S5	S7†	S1	S2	S5	S8‡	Nutrient	
II.1	−	−	−	−	+	+	−	+	+	r
II.3	−	−	−	−	+	+	−	+	+	r
II.4	−	−	−	−	+	+	−	−	+	c
II.5	+	+	+	+	+	+	−	−	−	w
II.6	+§	−	−	−	−	−	−	−	−	w§
II.7	+	+	+	+	+	+	−	−	−	w
II.8	+§	−	−	−	+	+	−	+	+	r+w§
II.9	−	−	−	+§	+	+	−	+	+	r+w§
Original culture 8370	+	+	−	+	+	+	−	+	+	c+r+w
No. 5										
II.12	+	+	+	−	+	+	+	−	−	w
II.13	+	+	+	−	+	+	+	−	−	w
II.14	+	+	+	−	+	+	+	−	−	w
II.16	+	+	+	−	+	+	+	−	−	w
II.17	+	+	+	−	+	+	+	−	−	w
II.19	+	+	+	−	+	+	+	−	−	w
Original culture no. 5	+	+	+	−	+	+	+	−	−	w

* Colony types: w=round colony which deposited sulphur; r=round colony which did not deposit sulphur; c=crenate-edged colony which did not deposit sulphur.

† Thiocyanate.

‡ Anaerobic.

§ Grew after 16 days in the liquid media only.

Second series of isolation experiments

In this series 8370 and no. 5 (a neopolitanus type organism which had been maintained in these laboratories for 5 months without showing any colony variation) were used. The characteristics of the stock cultures were tested immediately before isolation and ten isolates were made from each culture (Table 4). Of the ten isolates from 8370, seven grew within 1 week and each was a pure culture. After 2 weeks a further organism II.6 was found to be growing, and at this time II.8 and II.9 were found to contain two types of organisms giving no subculture r and w colonies. In each case the later occurring w colonies were shown to use thiocyanate and were I.4 of the previous experiment. Six of the isolated bacteria from no. 5 grew; each was a pure culture with the characteristics of the parent strain.

DISCUSSION

The conclusive evidence that these organisms may change comes from the first series of isolations in which four changes were observed; in two instances a single bacterium gave rise to a mixture of two organisms, and in one case a mixture of three occurred.

The second series confirmed these results with two more examples; but additional evidence is also available from the behaviour of the stock cultures of 'G' and 8370. The original culture of 8370 did not decompose thiocyanate, despite the many experiments which were made in attempts to confirm the findings of De Kruyff, van der Walt & Schwartz (1957) on this point. Four months later, when the isolations were made, two distinct organisms were found, each of which decomposed thiocyanate, neither of which could have been present in the original culture. In view of this evidence the authors believe that single organisms of some strains of thiobacilli can give rise to the range of organisms described in this paper.

The evidence against contamination is strong. The variants were never found in liquid or solid media exposed in the laboratory and the fact that culture no. 5 has not changed over the same period of time suggests that contamination is not the cause. Of the four stock cultures maintained in these laboratories two have changed and two have not, which suggests that the cause is not environmental, but is inherent in the organisms. This evidence suggests that the change is genetic rather than adaptive, especially when it is recalled that organisms which have arisen can utilize thiocyanate, to which they have not been exposed in the laboratory.

The media chosen for these experiments clearly distinguish four organisms. Two of these, I.4 and I.7, are strictly autotrophic thiobacilli, and I.6 corresponds to *Thiobacillus trautweinii*, which uses thiosulphate slowly under aerobic and anaerobic conditions. As the crenate organism did not use thiosulphate, it would not be classed as a thiobacillus, despite its close relationship to this group. Using the criteria given by Parker (1957), the organisms described may be regarded as distinct species; on these grounds inter-species change may be said to have occurred.

From the stock culture 'G', which was originally a strictly autotrophic strain, both heterotrophs and autotrophs have arisen. These results support the suggestion of Vishniac & Santer (1957) that there is little fundamental difference in this group between autotrophic and heterotrophic metabolism, and we have shown that these thiobacilli can change relatively easily from one mode of life to the other. This interconversion of species of *Thiobacillus* explains the results which we originally found with NCIB 8370. For the same reason, it is possible that De Kruyff, van der Walt & Schwartz (1957), using NCIB 8370, were working with I.7 organisms.

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Decomposition of Pyrimidines by *Nocardia corallina*

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SUMMARY

A bacterial species which degrades the pyrimidines, uracil, thymine and cytosine by induced enzymes has been characterized as *Nocardia corallina* (strain S). All other strains of *N. corallina* investigated oxidized thymine, but varied in their abilities to oxidize uracil and cystosine.

Organisms adapted to pyrimidines converted uracil to barbituric acid and thymine to 5-methylbarbituric acid. Oxidation of uracil by thymine-grown organisms was almost entirely by a pathway in which barbituric acid was an intermediate. Oxidation of thymine by uracil-grown organisms was similarly almost entirely via 5-methylbarbituric acid.

Oxidation of uracil by uracil-grown organisms and of thymine by thymine-grown organisms occurred, at least in part, through the respective barbituric acids. Discrepancies between the theoretical and observed values for O_2 uptake suggested however that other pathways may also occur in these cases.

Pyrimidine-grown organisms oxidized 2-thiouracil to 2-thiobarbituric acid and 2-thiothymine to a compound which was probably 5-methyl-2-thiobarbituric acid. These products were not further degraded by the organism.

Barbituric acid was oxidized by uracil-grown organisms to CO_2, NH_3 and urea with concurrent oxidative assimilation. The oxidation of barbituric acid was inhibited by isobarbituric acid and sodium azide although barbiturase activity in cell-free extracts was not affected by these substances. Barbiturase preparations converted barbituric acid anaerobically to malonic acid, CO_2 and NH_3, but barbituric acid was not degraded by whole organisms under anaerobic conditions. Whole organisms, grown on uracil, degraded urea but did not oxidize malonic acid. Acetic and propionic but not malonic or barbituric acids were activated by cell-free extracts as judged by hydroxamate formation. From the evidence presented, it is unlikely that free malonic acid is an intermediate in the breakdown of barbituric acid.

INTRODUCTION

Pyrimidine catabolism may be initiated either by a reduction yielding a dihydropyrimidine or by an oxidation giving a barbituric acid. The degradation of pyrimidines through dihydropyrimidines has been described in (*a*) animal tissues (Fink, Fink & Henderson, 1953; Fink, Cline, Henderson & Fink, 1956; Canellakis, 1956; Fritzson, 1957; Fritzson & Pihl, 1957); (*b*) *Pseudomonas aeruginosa* for uracil and thymine (Fink, Cline & Koch, 1954); (*c*) *Clostridium uracilicum* for uracil

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(Campbell, 1957*a–c*); (*d*) *Zymobacterium oroticum* and some species of *Corynebacterium* for orotic acid (Lieberman & Kornberg, 1953, 1954, 1955; Reynolds, Lieberman & Kornberg, 1955). A reductive pathway for uracil catabolism by *Torula utilis* was suggested from growth studies (Di Carlo, Schultz & Kent, 1952), but it is now known that dihydro-orotic acid is not attacked by this organism (Batt, Martin & Ploeser, 1953).

Pyrimidine catabolism with barbituric acids as intermediates has been demonstrated with certainty only in bacteria; species active in this respect have been isolated by the elective culture technique with pyrimidines as main source of carbon and nitrogen. The organisms obtained include a *Mycobacterium* sp. and a *Corynebacterium* (strain 161) (Hayaishi & Kornberg, 1952), a *Bacterium* sp. (Wang & Lampen, 1952*a*) and two strains of *Nocardia corallina* (Lara, 1952*a*; Batt & Woods, 1951). Results obtained, with both whole organisms and partially purified enzyme preparations, have led to the schemes for oxidative pyrimidine catabolism shown in Fig. 1. Barbituric acid was isolated as a product of uracil oxidation by whole organisms (Hayaishi & Kornberg, 1952), and this reaction has also been demonstrated with enzyme preparations from several different bacteria (Hayaishi & Kornberg, 1952; Wang & Lampen, 1952*b*; Lara, 1952*b*). The conversion of thymine to 5-methylbarbituric acid has been shown with enzyme preparations (Hayaishi & Kornberg, 1952), but the acid has not been clearly demonstrated to be an intermediate with intact organisms.

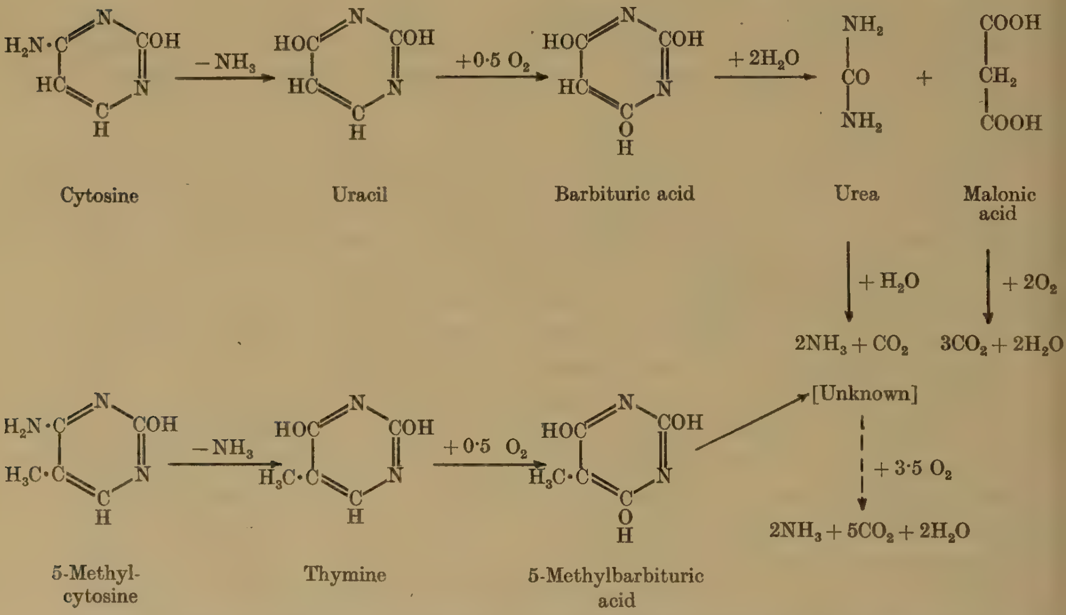

Fig. 1. Pathways of oxidative catabolism of pyrimidines (after Hayaishi & Kornberg, 1952).

Barbituric acid was hydrolysed to malonic acid and urea by extracts of uracil-adapted organisms of a strain of *Mycobacterium* (Hayaishi, 1952) and a similar enzyme (barbiturase) was described in cell-free extracts of *Nocardia corallina* grown on thymine (Lara, 1952*b*). Crude extracts of both these organisms showed urease activity and the products of the breakdown of barbituric acid were malonic acid, NH_3 and CO_2. Intact organisms of both species oxidized barbituric acid.

Barbiturase had no action on 5-methylbarbituric acid, 2-thiobarbituric acid or isobarbituric acid (Hayaishi & Kornberg, 1952). Intact organisms of the *Mycobacterium* sp. were tested for barbiturase activity by Hayaishi & Kornberg (1952) who stated that 'the removal of barbituric acid, which is carried out by cell-free enzymes at the same rate in the presence or absence of oxygen, does not occur under anaerobic conditions when intact cells are used'. The possibility was considered that energy may be required for the entry of barbituric acid into the organisms. Malonic acid was poorly utilized by uracil-adapted *Mycobacterium* sp. and although the organism may be impermeable to malonic acid, the possibility was suggested by Hayaishi & Kornberg (1952) that barbiturase produced from barbituric acid a labile derivative of malonic acid which was degraded to malonic acid by the isolation procedures.

The present paper describes the isolation and characterization of a bacterium able to utilize pyrimidines as the main source of carbon and nitrogen for growth. The main object of the work done with it was to investigate the possible pathways of degradation of a given pyrimidine when the organism was grown on the same or a different pyrimidine.

METHODS

Organisms. The bacterium with which most of the work was done was isolated from a sample of medium *A* (0·5 % KH_2PO_4, 0·2 % $MgSO_4,7H_2O$ and 0·2 % $CaCl_2$; pH 7·2) containing uracil (0·5 %) which had been exposed to the atmosphere for 12 hr. at room temperature (Batt & Woods, 1951). Following successive single colony isolations on tryptic meat digest agar, the organism was identified by Professor H. L. Jensen as a strain of *Nocardia corallina*; it will be referred to subsequently as strain S of this species.

The organism was aerobic, Gram-positive, non-motile, rod-shaped, non-sporulating, not acid-fast and it exhibited considerable pleomorphism when grown on different media. Neither acid nor gas was produced from glucose, sucrose, lactose, maltose, dulcitol or salicin, whereas acid but no gas was formed from sorbitol and mannitol. Gelatin was not liquefied and milk was not clotted; H_2S was produced by the organism growing in tryptic meat digest broth. By direct microscopy of young cultures (12–24 hr.) on agar small mycelia with granules were observed which were, possibly, the rudiments of aerial hyphae.

Four other strains of *Nocardia corallina* were made available by Professor Jensen and were designated strains P.F.M., 117, K_3 and Th_3. The growth conditions established for strain S were used for these organisms.

Growth tests. Medium *A* (5 ml.) supplemented with the test substance (20 mM) and Difco yeast extract (0·01 %) was distributed in 150 × 19 mm. tubes and autoclaved at 121° for 20 min. After inoculation with a dilute suspension of organisms (0·1 ml.; 0·05 mg. dry wt./ml.) harvested from a tryptic meat agar slope (30°; 24 hr.) the tubes were incubated at 30° in racks sloped at 5° above horizontal. Growth was estimated with an EEL photoelectric colorimeter (Evans Electroselenium Ltd., Halstead, Essex) with a neutral density filter. The relationship between dry wt. of organisms and instrument reading was linear up to a reading of 40; a reading of 20 was equivalent to 0·2 mg. dry wt. of organisms/ml.

Preparation of suspensions of organisms. For most experiments medium *A* (150 ml.) supplemented with a pyrimidine (1 g./l.) and yeast extract (Difco) (0·1 g./l.), was distributed in Roux bottles and autoclaved at 121° for 20 min. The inoculum was 0·75 mg. dry wt. of organisms harvested from a tryptic meat agar slope (24 hr.) The bottles were incubated horizontally in air at 30° for 65 hr. The organisms were harvested by centrifuging (2000 *g* for 20 min.), washed in the culture volume of 67 mM-phosphate buffer, pH 7·2, and resuspended in the same buffer at 10 mg. dry wt./ml.

For a few experiments the organisms were grown on tryptic digest of meat broth under the same conditions and with the same inoculum.

Preparation of cell-free enzyme preparations. A suspension of washed organisms (60 mg. dry wt. in 6 ml. of 67 mM-phosphate buffer, pH 7·2) was mixed with 4 g. ballotini beads (No. 13; Chance Bros., Smethwick, Staffs.), and 0·1 ml. of tributylcitrate, cooled in ice-water and shaken on a vibratory tissue disintegrator (H. Mickle, Gomshall, Surrey; Mickle, 1948) at maximum amplitude for 30 min. After centrifuging at 2500 *g* the straw-coloured supernatant fluid was stored at 0°.

Acetone powders were prepared by suspending 100 mg. dry wt. of organisms in 5 ml. of distilled water, chilling, and adding with vigorous stirring to acetone (50 ml.) previously cooled to −10°. The deposit after centrifuging was resuspended in acetone (20 ml.) and again centrifuged. After three further washings with acetone (10 ml.) the residue was freed from acetone by evacuating for 3 hr.; the yield of powder was 73 mg.

Estimations. Manometric estimations of O_2 consumption and CO_2 production were carried out by conventional methods (Umbreit, Burris & Stauffer, 1949). The manometer vessels contained 0·1 M-phosphate buffer (pH 7·2; 1·0 ml.), suspension of organisms (0·5 ml.) equivalent to 5 mg. dry wt. and distilled water (0·5 ml.) in the main compartment and 20 mM substrate solution (0·5 ml.) or distilled water (for the controls) in the sidebulb. The temperature was 30°.

For the estimation of non-gaseous products, the manometer vessels were chilled in ice-water, the contents poured into chilled tubes and centrifuged. The clear supernatant fluids were decanted and stored at 0°.

Pyrimidines were estimated spectrophotometrically. The samples were diluted 1 in 50 with either 0·1 N-HCl or 0·1 N-NaOH. The estimation of pyrimidines in binary mixtures followed the method described by Hotchkiss (1948) for determining purines in mixtures. The method of Markham & Smith (1949) was used for the separation and identification of the pyrimidines on paper chromatograms.

Ammonia was estimated in the presence of urea by distillation in the apparatus described by Markham (1942) after the addition of 0·35 M-borate buffer (pH 8·5). Urea was estimated by the difference in ammonia content of the solution before and after incubation with urease (Nimmo-Smith & Appleyard, 1956).

The method of Rose (1955) was used for the detection and estimation of hydroxamates formed from hydroxylamine. The enzyme preparation (0·5 ml.) was incubated in a total volume of 0·85 ml. with the substrate (340 μmole), adenosine triphosphate (3 μmole), Tris(2-amino-2-hydroxymethylpropane-1:3-diol; 100 μmole), $MgCl_2$ (10 mole) and hydroxylamine (930 mole); pH 7·7. After 30 min. at 30°, 10 % (w/v) trichloroacetic acid (1 ml.) was added and the mixture centrifuged. Ferric chloride solution (4 ml.; 1·25 % (w/v) in N-HCl) was added to the supernatant fluid and the colour intensity estimated in a photoelectric colorimeter using a 540 mμ filter.

Chemicals. 5-Methylbarbituric acid was synthesized by the method of Holmberg (1945) from diethylmethylmalonate and urea, the product obtained having a m.p. of 197° (uncorrected). 5-Hydroxymethyluracil was prepared by reacting formaldehyde with uracil in an alkaline solution (R. E. Cline, personal communication). Thymine glycol was synthesized by the method of Baudisch & Davidson (1925), and melted with decomposition at 210°. All other compounds used were obtained commercially.

RESULTS

Growth of the organism on pyrimidines

Nocardia corallina (strain S) grew in medium *A* containing either uracil, thymine, cytosine, 5-hydroxymethyluracil, barbituric acid or 5-methylbarbituric acid as the main source of carbon and nitrogen. In each case the lag phase was shortened by the addition to the medium of a small amount of yeast extract (Difco; 0·01 %) which also supplied a growth requirement of the organism for thiamine (Martin & Batt, 1957). The growth response to various pyrimidines of the five available strains of *N. corallina* were compared (Table 1); all grew well on thymine but they differed in their ability to utilize uracil and cytosine.

Table 1. *Growth of strains of* Nocardia corallina *on pyrimidines*

The medium (see Methods section) contained the pyrimidine stated (20 mM); incubated for 72 hr.

Strain of *N. corallina*	Growth (EEL reading) with		
	Uracil	Thymine	Cytosine
S	10	10	7
P.F.M.	7	11	3
227	3	11	4
K_3	1	15	2
Th_3	0	10	0

The substances thought by Cerecedo (1927, 1931) to be intermediates in the catabolism of uracil and thymine in dogs (dialuric acid, isodialuric acid, isobarbituric acid and thymine glycol) did not support the growth of strain S. The following substances also failed to support the growth of this organism on medium *A* supplemented with 0·01 % yeast extract (Difco): 5:6-dihydrouracil, 5:6-dihydrothymine, orotic acid, 2-thiothymine, 2-thiouracil, 2-thiobarbituric acid, 6-methyluracil, isocystosine, 2-aminopyrimidine, 2-amino-5-methyl-4-hydroxyprimidine, 5-amino-2:4-dihydroxypyrimidine, 4:6-dihydroxypyrimidine.

General action of suspensions of strain S on pyrimidines

Suspensions of organisms harvested after growth on either uracil or thymine rapidly oxidized uracil, thymine, barbituric acid and 5-methylbarbituric acid (Figs. 2, 3). Organisms grown on tryptic digest of meat broth oxidized all four pyrimidines only after a lag of at least 4 hr.; the enzyme systems are therefore induced by the presence of the substrate.

Organisms grown on the pyrimidines and tested on uracil, thymine and the barbituric acids completely removed the substrates, i.e. the rate of O_2 uptake decreased to values equal to those for the endogenous respiration and no pyrimidine remained.

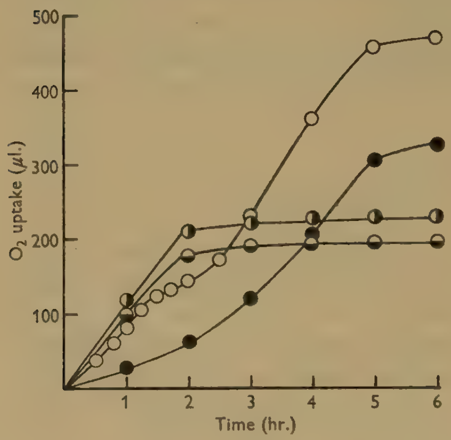

Fig. 2

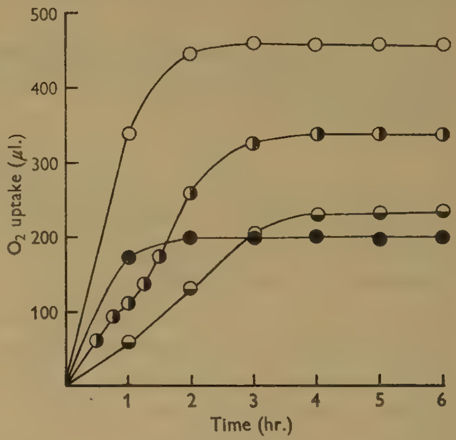

Fig. 3

Fig. 2. Uptake of oxygen by uracil-grown organisms (strain S) acting on thymine (○), 5-methylbarbituric acid (●), uracil (◑) and barbituric acid (◒). Conditions as described in Methods section with 10 μmole of substrate. All values corrected for endogenous O_2 uptake.

Fig. 3. Uptake of oxygen by thymine-grown organisms (strain S) acting on thymine (○), 5-methylbarbituric acid (●), uracil (◑) and barbituric acid (◒). Conditions as for Fig. 2.

The endogenous respiration of suspensions was high and, under the test conditions, amounted to about one-third of the O_2 uptake resulting from the degradation of the pyrimidine. Justification for subtracting the endogenous O_2 uptake from the experimental values in the presence of pyrimidines was obtained by determining the O_2 uptake with a constant amount of organism and different amounts of uracil; the corrected O_2 uptake values were directly proportional to the amount of substrate (Table 2).

Table 2. *Total oxygen uptake with varying amounts of uracil*

Uracil-grown organisms (strain S) incubated (as in Methods section) with the amount of uracil stated. The reaction was continued until the rate of O_2 uptake with uracil decreased to that with the organism alone. Corrected values are those in which the endogenous O_2 uptake value has been deducted.

Amount of uracil (μmole)	O_2 uptake			
	Corrected		Uncorrected	
	μmole	μmole/μmole uracil	μmole	μmole/μmole uracil
17·90	18·6	1·04	24·2	1·35
8·95	9·3	1·04	13·1	1·46
4·47	4·5	1·01	7·3	1·63

Urea, NH_3 and CO_2 were produced from uracil, thymine, barbituric acid and 5-methylbarbituric acid by organisms grown on either uracil or thymine. However, the values for the final O_2 uptake were much lower than the theoretical values calculated for the complete degradation of the pyrimidines to CO_2 and NH_3 (Table 3). No products other than urea, NH_3 and CO_2 were detected at the completion of the experiments of Table 3. The possibility that some of the substrate was undergoing oxidative assimilation was tested by carrying out the oxidations in the presence of sodium azide. With organisms grown on a given pyrimidine and tested on the same pyrimidine there was a marked increase in O_2 consumption although the theoretical value for complete oxidation was not reached (Table 4). With organisms grown on uracil and tested on thymine (and vice versa) even the lower concentration of azide used inhibited O_2 uptake (Table 4).

Table 3. *Total oxygen consumption during the oxidation of various pyrimidines*

Suspensions of organisms (strain S) grown on either uracil or thymine were incubated as described in the Methods section with 10 μmole of the stated pyrimidine. The O_2 uptake values have been corrected for the endogenous uptake. The theoretical O_2 uptake values were calculated for the complete conversion of the pyrimidines to CO_2 and NH_3.

	O_2 uptake (μmole/μmole pyrimidine) for organisms grown on		Theoretical O_2 uptake (μmole/μmole pyrimidine)
Substrate	Uracil	Thymine	
Uracil	1·01	1·48	2·5
Barbituric acid	0·87	1·03	2·0
Thymine	2·07	2·05	4·0
5-Methylbarbituric acid	1·47	0·88	3·5

5:6-Dihydrouracil and 5:6-dihydrothymine were not oxidized by suspensions of organisms grown on uracil and thymine respectively.

Table 4. *Effect of sodium azide on the oxygen uptake with uracil and thymine*

Organisms grown as stated on either uracil or thymine were incubated as described in the Methods section with either uracil or thymine in the presence of the stated concentration of sodium azide. The reaction was normally continued until the O_2 uptake decreased to the endogenous rate; * however indicates that the results were calculated after 5 hr. incubation even though the rate was still above the endogenous value.

	O_2 uptake (μmole/μmole pyrimidine) with			
	Uracil-grown organisms oxidizing		Thymine grown organisms oxidizing	
Conc. of azide (mM)	Uracil	Thymine	Uracil	Thymine
0	1·07	2·12	1·43	1·90
5	1·92	0·62	1·06	2·82
20	1·5*	0·49	0·47	2·5*
Theoretical value for complete oxidation	2·5	4·0	2·5	4·0

Oxidation of uracil and thymine to the corresponding barbituric acids

Thymine oxidation by organisms grown on uracil. The rate of uptake of O_2 showed a sudden change at a value of approximately 0·5 mole O_2/mole thymine (Figs. 2, 4). After this time no thymine remained but a compound was present which absorbed ultraviolet radiation. At the end of the first phase of thymine oxidation (about 60 min.) 0·12 mole CO_2 and 0·09 mole NH_3/mole thymine were present but only traces of urea were detected (Fig. 4). The oxidation product of thymine had identical $\lambda_{max.}$ values in 0·1 N-HCl (262 mμ) and 0·1 N-NaOH (268 mμ) to those for synthetic 5-methylbarbituric acid. On the assumption that the oxidation product is 5-methylbarbituric acid, the amount present at the point of inflexion of the O_2 uptake curve is equivalent to 95–100 % conversion of the added thymine to the acid. Both disappearance of thymine and production of 5-methylbarbituric acid were complete at the time O_2 consumption reached 0·5 mole/mole thymine originally present (Fig. 4).

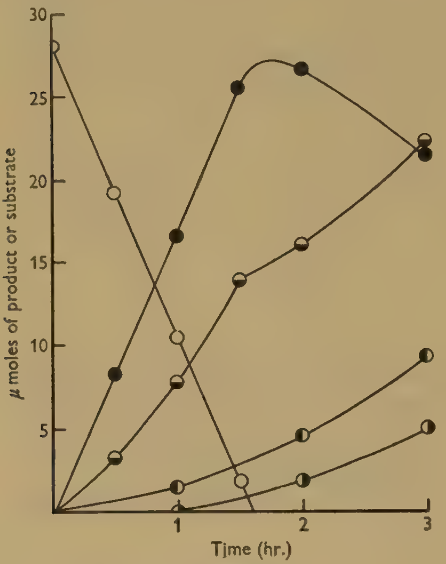

Fig. 4

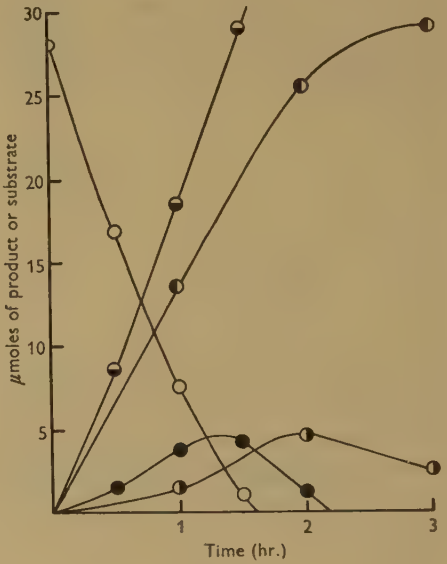

Fig. 5

Fig. 4. Metabolism of thymine (28 μmole) by organisms grown on uracil. Thymine (○), 5-methylbarbituric acid (●), O_2 uptake (◒), NH_3 (◐) and urea (◑). All values corrected for control values without substrate.

Fig. 5. Metabolism of thymine (28 μmole) by organisms grown on thymine. Symbols as for Fig. 4.

The primary oxidation product of thymine was isolated and characterized as 5-methylbarbituric acid as follows. Uracil-grown organisms were incubated with thymine in a number of manometer vessels until the change in rate of O_2 uptake occurred. After removing the organisms by centrifuging, the supernatant fluid was evaporated to dryness under vacuum. The residue was dissolved in a minimum volume of water and 30 % (w/v) $CaCl_2$ added until no further precipitation occurred. The filtrate obtained after removal of the calcium phosphate was evaporated to dryness and the residue extracted with β-ethoxyethanol; the extract was again evaporated. Crystallization from ethanol yielded a white solid (30 mg., m.p. 193°) which gave a mixed melting-point with authentic 5-methylbarbituric acid (m.p. 197°) of 195°. The absorption maximum (262 mμ in 0·1 N-HCl and 268 mμ in 0·1 N-NaOH) and R_F values (Table 5) were identical with the values for 5-methylbarbituric acid.

The curve for O_2 uptake with 5-methylbarbituric acid was almost identical to the curve for the second phase of thymine oxidation (Fig. 2). 5-Methylbarbituric acid is spontaneously and rapidly oxidized in air, under both acid and neutral conditions, to 5-hydroxy-5-methylbarbituric acid (Nishikawa, 1931; Stuckey, 1942). When 5-methylbarbituric acid (10 μmole) in phosphate buffer (0·08 M; pH 7·2) was shaken aerobically in a manometer vessel, the theoretical O_2 uptake for conversion to 5-hydroxy-5-methylbarbituric acid was reached in 30 min. However, when 5-methylbarbituric acid was incubated with a suspension of organisms (Fig. 1) in a solution of the same phosphate concentration and pH, the rapid conversion to 5-hydroxy-5-methylbarbituric acid did not occur; the products of the oxidation in the presence of the organism were CO_2, NH_3 and urea. 5-Methylbarbituric acid was detected in the reaction mixture throughout the period of oxidation (5 hr.). The presence of the actively metabolizing suspension of organisms effectively suppressed the formation of 5-hydroxy-5-methylbarbituric acid; the latter compound was not itself oxidized by the organism.

Table 5. *Chromatography of pyrimidines and products formed from them by strain S*

Solvent systems: (A) *n*-butanol saturated with water, (B) isopropanol + water (100:30), (C) isopropanol + water ammonia; the same solvent as (B), a beaker containing ammonia (sp.gr., 0·88) being placed in the chromatography tank, (D) isopropanol + water + HCl (10 N) (680:156:164).

All of the oxidation products showed as yellow spots on chromatograms sprayed with 0·1% methyl red in borate buffer pH 8·5 indicating that they were all acidic compounds.

	R_F with (solvent system)			
	(A) Butanol + water	(B) Isopropanol + water	(C) Isopropanol + water + ammonia	(D) Isopropanol + water + HCl
Pyrimidine				
Uracil	0·35	0·60	0·54	0·67
Thymine	0·51	0·69	0·64	0·76
2-Thiouracil	0·57	0·66	0·58	0·70
2-Thiothymine	0·69	0·75	0·67	0·79
Barbituric acid	0·05	0·20	0·43	0·49†
5-Methylbarbituric acid	0·05	0·23	0·49	—*
2-Thiobarbituric acid	0·05	0·26	0·48	0·66†
Oxidation product formed from				
Uracil	0·05	0·21	0·44	0·49†
Thymine	0·05	0·24	0·50	—*
2-Thiouracil	0·05	0·25	0·47	0·66†
2-Thiothymine	0·08	0·30	0·58	—*

* 5-Methylbarbituric acid spots were obtained with acid solvents only when relatively large quantities were chromatographed. The compound formed from thiothymine resembled 5-methylbarbituric acid in being unstable in acid solvents.

† The barbituric acids showed as yellow spots on chromatograms run with isopropanol + water + HCl. The oxidation product from thiouracil also showed as a yellow spot on the chromatograms.

Thymine oxidation by organisms grown on thymine. In this case O_2 consumption was more rapid and no sudden change in rate occurred (Figs. 3, 5). Only a small amount of 5-methylbarbituric acid accumulated transiently during the incubation (Fig. 5); the same was true of urea, but NH_3 was formed rapidly from the outset.

Uracil oxidation by organisms grown on thymine. A change in the rate of O_2 uptake was observed at a point corresponding to 0·5 mole O_2/mole uracil (Fig. 3). After this point, no uracil remained but a compound had accumulated which absorbed ultraviolet radiation. This compound disappeared slowly on further incubation (Fig. 6). The absorption characteristics of the compound were identical in 0·1 N-HCl ($\lambda_{max.}$ 257 mμ) and 0·1 N-NaOH ($\lambda_{max.}$ 259 mμ) to the values for barbituric acid; the R_F values for the oxidation product determined on paper chromatograms with several solvent systems were the same as the values for barbituric acid (Table 5).

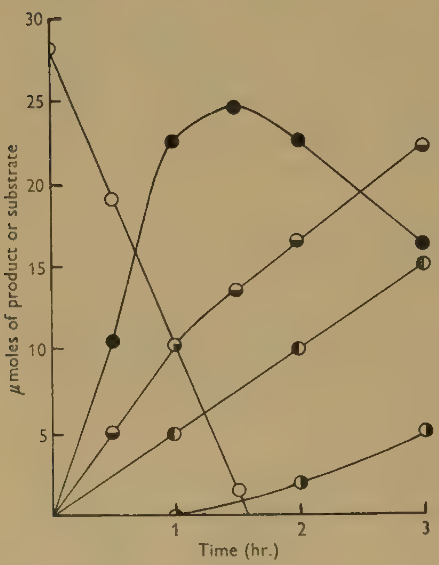

Fig. 6

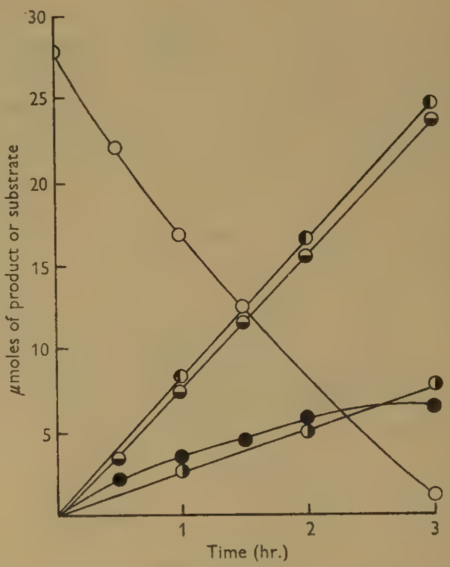

Fig. 7

Fig. 6. Metabolism of uracil (28 μmole) by organisms grown on thymine. Uracil (○), barbituric acid (●), O_2 uptake (◒), NH_3 (◐) and urea (◑). All values corrected for control values without substrate.

Fig. 7. Metabolism of uracil (28 μmole) by organisms grown on uracil. Symbols as for Fig. 6.

At its maximum the amount of barbituric acid formed was equivalent to about 90 % of the uracil added (Fig. 6). Formation of NH_3 was linear from the outset, but urea was only detected after 2–3 hr. The rate of O_2 uptake in Fig. 6 is for 28 μmole of uracil added initially; when the ratio of amount of organisms to pyrimidine concentration was reduced, as in this experiment, the change in rate of O_2 uptake was usually less pronounced than that shown in Fig. 3.

Uracil oxidation by organisms grown on uracil. As with the oxidation of thymine by thymine-grown organisms no abrupt changes in the rate of O_2 consumption were observed during the incubation (Figs. 2, 7). Accumulation of barbituric acid was small and transitory (Fig. 7). Urea and NH_3 production were both linear but the latter was the more rapid.

Oxidation of 2-thiouracil and 2-thiothymine

During a search for compounds capable of inhibiting pyrimidine catabolism it was found that strain S, grown in the presence of either uracil or thymine, oxidized 2-thiouracil and 2-thiothymine with an overall O_2 uptake of 0·5 mole O_2/mole of 2-thiopyrimidine removed. The products of thiopyrimidine oxidation absorbed ultraviolet radiation. During the oxidation of 2-thiothymine there was a change in the $\lambda_{max.}$ value (pH 7·2) from 275 to 272 mμ, while with 2-thiouracil as substrate the change was from 270 to 265 mμ; the $\lambda_{max.}$ for 2-thiobarbituric acid at pH 7·2 is 265 mμ. The rates at which 2-thiouracil and 2-thiothymine were oxidized were approximately equal to the rates of oxidation of uracil or thymine by the same bacterial suspension.

The oxidation products of 2-thiouracil and 2-thiothymine were chromatographed in various solvents and the R_F values compared (Table 5) with different reference compounds including the products formed when the organism oxidized uracil and thymine. The acidic nature of the compounds formed from 2-thiouracil and 2-thiothymine was demonstrated by spraying the chromatograms with a buffered solution of methyl red. When the oxidation of 2-thiouracil or 2-thiothymine by thymine-grown organisms was carried out in unbuffered medium the pH fell rapidly (for 2-thiothymine from 6·8 to 4·0) and only a small O_2 uptake was observed. The results indicate that the product formed from 2-thiothymine was 2-thiobarbituric acid and that 2-thiothymine, by analogy, was probably converted to 2-thio-5-methylbarbituric acid; an authentic sample of the last-named substance was not available. As these reactions were only observed with pyrimidine-adapted organisms, it was concluded that the oxidations were catalysed by the enzyme systems induced by uracil and thymine. 2-Thiobarbituric acid (and presumably 2-thio-5-methylbarbituric acid) was not attacked by pyrimidine-adapted suspensions of the organism.

Catabolism of barbituric acid

Effect of growth medium. Barbituric acid was oxidized immediately and rapidly to CO_2, NH_3 and urea by suspensions of strain S which had been grown on uracil, thymine or barbituric acid, whereas when grown in media containing no added pyrimidines no oxidation occurred until after a lag of about 8 hr. It appears that barbituric acid, uracil and thymine can all induce the enzymes required for the degradation of barbituric acid. The O_2 uptake curves obtained when organisms grown on barbituric acid were tested with uracil, thymine, barbituric acid and 5-methyl-barbituric acid (Fig. 8) were similar to those obtained when uracil-grown organisms oxidized these four pyrimidines (fig. 2) suggesting that barbituric acid can also induce enzymes for the initial attack on uracil and thymine.

Requirement for oxygen. Suspensions of the organism (grown on uracil) had no action on barbituric acid in an atmosphere of N_2.

Products of barbituric acid catabolism. The equations for the oxidation of barbituric acid by suspensions of strain S (1) and for the complete oxidation of barbituric acid to CO_2 and NH_3(2) are:

$$C_4H_4N_2O_3 + 0{\cdot}88O_2 \rightarrow 2{\cdot}52CO_2 + 0{\cdot}72NH_3 + 0{\cdot}36CO(NH_2)_2, \tag{1}$$

$$C_4H_4N_2O_3 + 2O_2 + H_2O = 4CO_2 + 2NH_3. \tag{2}$$

The rates of production of ammonia and urea from barbituric acid by uracil-grown organisms were compared with the rate of removal of the acid and the rate of O_2 uptake (Fig. 9). The total O_2 uptake values were reproducible to within 10 %; for six experiments the values (corrected for the endogenous O_2 uptake) were 0·89, 0·85, 0·89, 0·81, 0·90 and 0·92 μmole O_2/μmole barbituric acid removed. The incomplete recovery of carbon and nitrogen (as CO_2, NH_3 and urea) suggested the possibility that concurrent oxidative assimilation was occurring during the oxidation of barbituric acid.

Effect of sodium azide. An increase of 54 % in the total O_2 uptake was obtained when barbituric acid was oxidized by suspensions of the organism in the presence of 2 mM-sodium azide; this was the largest increase observed with the range of concentrations (0·1 mM–0·2 M) of azide tested.

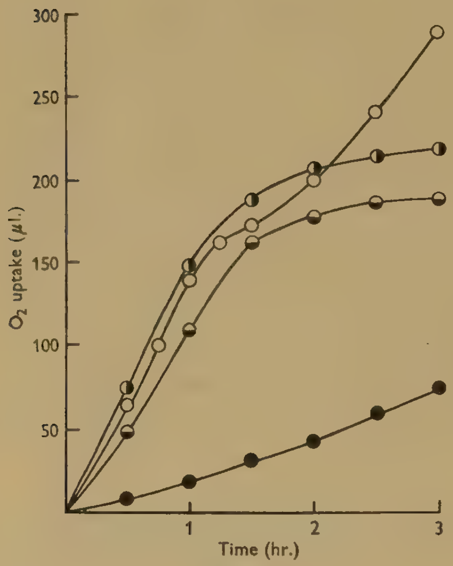

Fig. 8

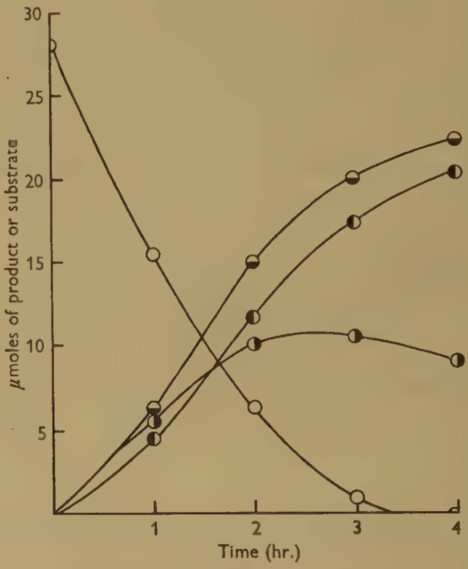

Fig. 9

Fig. 8. Uptake of oxygen by barbituric acid-grown organisms acting on thymine (○), 5-methylbarbituric acid (●), uracil (◑) and barbituric acid (◒). The amount of each substrate was 10 μmole. All values corrected for endogenous O_2 uptake.

Fig. 9. Metabolism of barbituric acid by organisms grown on uracil. Barbituric acid (○), O_2 uptake (◒), NH_3 (◐) and urea (◑). All values corrected for control values without substrate.

High concentrations of azide (0·2 M) completely inhibited the oxidation of barbituric acid by uracil-grown organisms and also caused barbituric acid to accumulate in the reaction mixture when similar organisms were oxidizing uracil. With thymine-grown organisms acting on uracil in the presence of 0·2 M-azide, the accumulation of barbituric acid was equivalent to the amount of uracil which had disappeared. The activity of the enzymes catalysing the initial attack on uracil and thymine was not affected by azide at this concentration.

Effect of isobarbituric acid. During a search for compounds which might be either intermediates in barbituric acid catabolism or inhibitors of its utilization, isobarbituric acid was found almost completely to suppress the oxidation of an equimolar concentration of barbituric acid (Table 6).

Degradation by cell-free extracts. Extracts made from uracil-grown organisms with the Mickle tissue disintegrator decomposed barbituric acid anaerobically to NH_3, CO_2 and malonic acid; 9 μmole of barbituric acid was removed by 1 ml. of enzymic extract in 1 hr. A similar extract from thymine-grown organisms had only one-quarter of the activity. Malonic acid (50 mg.) was isolated by the method of Hayaishi (1952) from the products of a large-scale experiment in which enzymic extract (8 ml.) was incubated with barbituric acid (200 mg.) in 12 ml. of 0·2 M-phosphate buffer (pH 7·2) in the presence of toluene (0·5 ml.) at 30° until the barbituric acid had been decomposed (28 hr.). The enzyme catalysing the anaerobic breakdown of barbituric acid will be referred to as barbiturase; the crude preparation used above also contained urease.

Table 6. *Effect of isobarbituric acid on pyrimidine catabolism*

Organisms grown on either uracil or thymine as indicated were incubated for 3 hr. with uracil or barbituric acid (each 10 μmole) in the absence or presence of isobarbituric acid (10 μmole). After removing the organisms by centrifuging, the supernatant fluids were analysed for pyrimidines.

	Pyrmidines (μmole) formed or removed by			
	Uracil-grown organisms with isobarbituric acid		Thymine-grown organisms with isobarbituric acid	
	Absent	Present	Absent	Present
Uracil removed	10	6·6	10	10
Barbituric acid removed	10	1	10	1
Barbituric acid formed from uracil	0	1	0	5

Sodium azide and isobarbituric acid, which both inhibited the oxidation of barbituric acid by whole organisms, were without action on the activity of barbiturase in the cell-free extracts.

Urea was degraded by intact organisms, acetone powders and extracts made with the Mickle tissue disintegrator. It is likely that the primary products of barbiturase action are malonic acid and urea, and that the latter is then decomposed to NH_3 and CO_2 by the urease present in the preparations.

Metabolism of dicarboxylic acids

Several dicarboxylic acids were tested as possible sources of carbon for aerobic growth in medium *A* supplemented with phosphate buffer (0·1 M; pH 7·2), thiamine (3 mM) and $(NH_4)_2SO_4$ (25 mM); the carbon sources were added, as the sodium salts, at a final concentration of 0·1 mM. Succinic, malic, fumaric and methylmalonic acids supported the growth of the organism but no growth was obtained with malonic, ketomalonic and tartronic acids.

Suspensions of uracil-grown organisms did not oxidize malonic acid. In addition malonic acid had no effect on the oxidation of uracil or barbituric acid by such organisms.

Formation of hydroxamic acids by cell-free extracts. Hayaishi (1955) showed that malonic acid was degraded to acetic acid by cell-free enzyme preparations of *Pseudomonas fluorescens* with the intermediate formation of coenzyme A derivatives of malonic acid and acetic acid. If these two acids are intermediates in the aerobic catabolism of barbituric acid by strain S it is possible that the organism would contain enzyme systems for their activation and conversion to coenzyme A derivatives. Activation was tested by the method of Rose (1955) by studying the formation of hydroxamates by cell-free extracts in the presence of hydroxylamine and adenosine triphosphate; a number of other carboxylic acids were also tested (Table 7). The enzymic extract contained activation systems for acetic and propionic acids but not for malonic acid or the other substances tested.

Table 7. *Formation of hydroxamates by cell-free extracts of strain S*

Cell-free extracts were prepared with the Mickle tissue disintegrator and incubated with the test substrates as described in the Methods section.

Substrate	Colorimeter reading
None	0
Acetate	12
Propionate	22
Malonate	0
Barbiturate	0
Uracil	0
Lactate	1
Succinate	2
β-Hydroxypropionate	0

Oxidation of acetic acid. Since acetic acid or a derivative (acetyl coenzyme A) was considered as a possible intermediate in barbituric acid catabolism its oxidation by suspensions of strain S was studied in the presence and absence of sodium azide. It was oxidized rapidly and without lag, irrespective of the nature of the growth medium from which the organism had been harvested. With organisms grown on uracil, the final O_2 uptake was 1·04 mole O_2/mole acetate and concurrently, 1·1 mole CO_2/mole acetate was produced. The oxidation of acetic acid was partially uncoupled by azide (2 mm) which gave an increase from 1·04 to 1·66 mole O_2/mole acetate removed; the theoretical value for complete oxidation is 2 mole O_2/mole acetate.

Analysis of medium after growth

It was considered possible that strain S metabolized barbituric acid by two pathways, and that one of these, catalysed by barbiturase, yielded malonic acid which was not further utilized by the organism. The possibility was tested by determining whether malonic acid accumulated during growth on uracil. Organisms were grown on uracil as described for the preparation of washed suspensions except that a sample of sterile medium was removed before inoculation for pyrmidine analysis. After culture for 65 hr. the organisms were removed by centrifugation and the supernatant fluid analysed for pyrimidines, corrections being made for evaporation of water during the incubation period. The amount of uracil removed was 54·5 % of that initially present and barbituric acid was formed in amounts equivalent to 5·5 % of the uracil added.

The supernatant fluid was evaporated to a small volume, acidified with $3\text{N-}H_2SO_4$ and continuously extracted with ether for 48 hr. The very small amount of material extracted by the ether contained no malonic acid as indicated by chromatography on paper. No substances could be sublimed from the ether extract.

Pyrimidine oxidation by strain K_3

Nocardia corallina strain K_3 grew much better on thymine than on uracil or cytosine (Table 1). Suspensions of thymine-grown organisms rapidly oxidized both thymine and 5-methylbarbituric acid and uracil was converted to barbituric acid (Table 8). Barbituric acid was metabolized only slowly.

Table 8. *Pyrimidine catabolism by suspensions of strain K_3*

Organisms grown on thymine and incubated aerobically in manometer vessels (as described in Methods section) with 10 μmole of the substrate indicated.

	Substrate			
	Uracil	Thymine	Barbituric acid	5-Methylbarbituric acid
Substrates removed (μmole)	8·0	8·8	1·5	10·0
Barbituric acid formed (μmole)	7·0	—	—	—
5-Methylbarbituric acid formed (μmole)	—	8·0	—	—

DISCUSSION

The long lag period which elapsed before pyrimidines were attacked by suspensions of *Nocardia corallina* from medium containing no added pyrimidine makes it clear that the enzyme systems required for the overall oxidative metabolism of pyrimidines by strain S were induced by the presence of the substrate. Either uracil or thymine can induce the enzyme(s) for an initial attack on both pyrimidines, yielding the corresponding barbituric acids, and this enzyme will be referred to as the uracil-thymine oxidase (Hayaishi & Kornberg, 1952). Growth on either uracil or thymine also induced in the organism ability to oxidize (probably to the corresponding barbituric acids) both 2-thiouracil and 2-thiothymine and it is probable that uracil-thymine oxidase also catalyses these reactions. A cell-free preparation of a similar enzyme from a *Mycobacterium* sp. was found by Hayaishi & Kornberg (1952) not to oxidize 2-thiouracil.

Since neither 5:6-dihydrouracil nor 5:6-dihydrothymine supported growth of strain S and were not oxidized by washed suspensions it is unlikely, unless these substances do not enter the organism, that a preliminary reduction (see Introduction) is an initial step in the overall oxidation of uracil and thymine.

The O_2 uptake with thymine and uracil by organisms grown with either pyrimidine was considerably less than the values required for complete oxidation to CO_2 and NH_3, but no other product apart from urea (whose conversion to CO_2 and NH_3 requires no O_2) was detected. It is probable therefore that extensive oxidative assimilation of the substrate into cell material was taking place. However, O_2 uptake was increased by an uncoupling agent (sodium azide) only when organisms were oxidizing the pyrimidine on which they had been grown and not, for example, when thymine-grown organisms were oxidizing uracil (Table 4). The failure to demonstrate oxidative assimilation by the use of azide in the latter case may be explained as follows. It may be assumed that organisms grown on uracil contain fully developed all the enzymes required for the complete oxidation of uracil. On the other hand, organisms grown on thymine are adapted for the conversion of uracil to barbituric acid, but the enzymes for the degradation of the latter are not fully induced and barbituric acid at first accumulates almost quantitatively (Fig. 6). It is suggested that azide inhibits the formation of the enzymes which metabolize barbituric acid and give rise to the intermediates used in oxidative assimilation. This view is supported by the observation that barbituric acid itself was oxidized by uracil-grown organisms and that the oxidation was partly uncoupled from assimilation by azide; barbituric acid was not however oxidized by thymine-grown organisms in the presence of azide.

Similar arguments apply to the converse case in which oxidative assimilation could not be demonstrated with uracil-grown organisms metabolizing thymine. It is concluded that failure to demonstrate oxidative assimilation with azide in these two cases does not mean that such assimilation does not occur.

When uracil was oxidized by organisms grown on thymine there was an initial and almost theoretical accumulation of barbituric acid (Fig. 6) which was later further oxidized (Fig. 3); the bulk of uracil metabolism therefore occurred via barbituric acid. With uracil-grown organisms, however, barbituric acid was present in only small amounts at any stage of the reaction. The results might be explained on the assumption that the enzymes for further metabolism of barbituric acid are not fully developed in thymine-grown organisms; the initial rate of O_2 uptake with barbituric acid was only one-third that with 5-methylbarbituric acid (Fig. 3). However, if the whole of the uracil was also metabolized by uracil-grown organisms through the intermediate formation of barbituric acid it would be expected that the O_2 consumption with barbituric acid should be 0·5 mole less than that with uracil, i.e. by the amount of O_2 required for the conversion of uracil to barbituric acid. Experimentally the difference was only 0·15 mole O_2 (Table 3).

The position with regard to oxidation of thymine by organisms grown on uracil and thymine, respectively, was analogous. 5-Methylbarbituric acid was established as an intermediate with certainty only with uracil-grown organisms. With thymine-grown organisms a little of the acid accumulated, but the O_2 uptake values for thymine and 5-methylbarbituric acid differed by almost 1·2 mole instead of the expected 0·5 mole. It is concluded therefore that in the case of the oxidation of uracil and thymine by organisms grown on uracil and thymine, respectively, there is a possibility that a second pathway exists in which the corresponding barbituric acids are not intermediates.

There is evidence from work with cell-free extracts of various bacteria (Hayaishi & Kornberg, 1952; Lara, 1952*b*) that barbituric acid is initially attacked by an enzyme, barbiturase, which degrades it anaerobically to malonic acid and urea. The same enzyme is present in cell-free extracts of the present organism but intact organisms attacked barbituric acid only when O_2 was present. Furthermore isobarbituric acid and sodium azide inhibited completely the metabolism of barbituric acid by whole organisms, but did not affect the barbiturase activity of cell-free extracts. The possible role of barbiturase in the aerobic metabolism of barbituric acid by whole organisms is therefore not clear; some of the difficulties have been considered by Hayaishi & Kornberg (1952). Suspensions of strain S harvested from growth on either barbituric acid or uracil rapidly oxidized barbituric acid to CO_2, NH_3 or urea with considerable concurrent oxidative assimilation.

Hayaishi (1955) showed the presence in *Pseudomonas fluorescens* of enzymes for the conversion of malonic acid to a coenzyme A derivative and for the conversion of the latter to acetic acid through the intermediate formation of acetyl coenzyme A. Evidence for the activation of malonic acid by cell-free extracts of strain S was sought by a study of hydroxamate formation but none was found; acetate was activated under the same conditions. Malonate was not oxidized by pyrimidine-adapted suspensions of strain S nor could it be detected in the medium after growth of the organism on uracil. The possibility exists that an active derivative of malonate is formed directly from barbituric acid and that this is immediately metabolized without intermediate conversion to free malonate. It is also possible that malonate does not permeate the organism, though this would not explain the lack of activation by cell-free extracts.

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Morphogenesis of Mycoplasma and Bacterial L-form Colonies

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SUMMARY

The development and mechanism of formation of Mycoplasma and bacterial L-form colonies were studied. The micro-organisms penetrate into the agar within a few hours after inoculation. It is suggested that penetration is caused by capillary forces which draw the minute plastic organisms into the dried agar gel, together with the water surrounding them. Penetration does not take place when the agar surface is very moist. The organisms appear to develop in the interstices of the fibrillar network of the agar gel and form a firm and elastic ball-like colony growing in all directions. When reaching the agar surface the growth spreads into the thin free water film which covers the agar, forming the peripheral zone typical of mature colonies. Factors which decrease the thickness of the free water film, like drying the medium or increasing the agar concentration, cause a decrease of the peripheral zone dimensions. Factors which retard growth, such as high concentrations of inorganic salts or hydrogen ions, inhibit the formation of the peripheral zone by limiting the initial ball-like growth inside the agar, rendering it incapable of reaching the agar surface. Typical colonies composed of both central and peripheral zones appear also on other fibrillar media such as the cellulose pellicle of *Acetobacter xylinum* and gelatin.

INTRODUCTION

Mycoplasma organisms (PPLO) and the L-forms of bacteria share a typical colony morphology, the 'fried-egg' colony. This colony is made up of a central zone, embedded in the agar and a peripheral zone on the agar surface (Klieneberger-Nobel, 1960; Dienes, 1960). The morphology of the colony, unique for these organisms, serves as one of the most important criteria in distinguishing them from other bacteria. Although much work has been done on the morphology of Mycoplasma and L-forms, the information concerning the mode of formation of the colonies is scanty (Klieneberger, 1934; Dienes, 1945). Our aim was, therefore, to study the development of these peculiar colonies and the mechanisms involved in their formation.

Two features shared by the Mycoplasma and L-forms seem to be responsible for the formation of the 'fried-egg' colonies: the very small dimensions (100–300 mμ) of the minimal reproductive units (Klieneberger-Nobel, 1956) and their plasticity due to the absence of a rigid cell wall (Klieneberger-Nobel, 1960).

METHODS

Organisms. Mycoplasma laidlawii strain A (PG8), *M. laidlawii* strain B (PG9), *M. bovigenitalium* (PG11), *M. gallisepticum* (PG31), *M. mycoides* var. *mycoides* (PG1), *M. neurolyticum* (PG 28) and the stable L-form of *Proteus* were kindly given by Dr D. G. ff. Edward (The Wellcome Research Laboratories, Beckenham, Kent). *M. mycoides* var. *capri* and the stable L-form of *Streptobacillus moniliformis* were the gift of Dr E. Klieneberger-Nobel (The Lister Institute for Preventive Medicine, London). *M. hominis* was isolated in our laboratory from a case of vaginitis.

Media. The medium used for stock cultures and most experiments was a modified Edward medium (Butler & Knight, 1960*a*) where pooled inactivated human serum replaced horse serum. Beef heart for infusion, peptone and yeast extract used for compounding Edward medium were products of Difco Laboratories Inc. (Detroit 1, Michigan, U.S.A.). Following the suggestion of Klieneberger-Nobel (1956) the medium was enriched by addition of a nutrient broth culture filtrate of *Staphylococcus aureus* in a final concentration of 12·5 % (v/v). Edward medium was solidified with 1·25 % (w/v) Bacto-agar (Difco, certified) except for some experiments where 12 % (w/v) Bacto-gelatin (Difco) was used.

Coagulated serum medium (Löffler's medium) was prepared by mixing three parts of bovine serum with one part of Difco nutrient broth containing 1 % (w/v) glucose. The medium was poured into Petri dishes and sterilized by inspissation at 85° for 2 hr.

Cellulose medium. Acetobacter xylinum was grown on the medium of Hestrin & Schramm (1954) for 2 days at 30°. The thick pellicle formed was removed, washed thoroughly with sterile distilled water and immersed in sterile distilled water at 6° for 3 days. The water was changed daily. The white cellulose pellicle was then soaked in liquid Edward medium, transferred to a filter paper disk to remove excess liquid and finally placed on the surface of solid Edward medium.

Conditions of growth. Test tubes ($6 \times \frac{5}{8}$ in.) containing 5 ml. of liquid Edward medium were inoculated with 0·1 ml. of the stock cultures and incubated statically in air at 37°. Optimal growth of all strains occurred usually after 48 hr. incubation. Decimal dilutions of these broth cultures were made in a solution of 1 % (v/v) Edward medium in 0·01 M-K_2HPO_4 buffer (pH 8·0; Butler & Knight, 1960*b*). Standard drops (0·02 ml. each) of these dilutions were placed on solid medium plates which had previously been dried by leaving them open in the 37° incubator for 1–1·5 hr. The dilutions used for inoculation were chosen so as to give no more than 500 colonies per drop area. The plates were placed in closed tins containing moist cotton wool and incubated at 37° for 3 days.

Measurement of colony and central zone diameters. The growth which appeared on plates was inspected by means of an ordinary light microscope, using slightly oblique transmitted light. Measurement of colony and central zone diameters was carried out with a ×10 objective and a ×5 ocular fitted with a micrometer disk. The drop area was divided by a line through its centre cut by means of a razor blade, and 50 colonies were measured along this line, assuring the representation of colonies growing in the periphery and centre of the drop area.

Vertical sections of colonies. Large colonies were selected for this purpose. The section was cut through the centre of the colony by means of a razor blade. For good results the thickness of the section had to be not greater than 0·2–0·3 mm. The sections were transferred to slides and examined by the light microscope.

Technique for demonstrating penetration of micro-organisms into the agar. Standard drops of diluted suspensions of the organisms tested were placed on the surface of dried Edward medium plates. Immediately after the drops had dried and at various time intervals later, small agar squares (larger than a drop area) were cut from the Edward medium and placed on the drop areas, care being taken not to form air bubbles. The agar slices were removed, turned over and placed on slides. The agar plates and slices were then incubated at 37° for 3 days in a moist atmosphere. The number of colonies appearing in the drop areas on the plates and on the corresponding agar slices was determined by using the light microscope (magnification × 25).

RESULTS

The development of Mycoplasma and bacterial L-form colonies

Edward medium plates were inoculated with suspensions of *Mycoplasma laidlawii* strain A and the L-form of *Streptobacillus moniliformis*. Visible colonies appeared after 24-hr. incubation at 37° (Pl. 1, figs. 1, 4). At this phase of growth almost all colonies in both organisms appeared to be small spheres embedded in agar. This was proved by the circular shape of vertical sections through the colonies and by the inability to remove the colonies on scraping the agar surface with a razor blade.

Further incubation for another 24 hr. caused enlargement of *Mycoplasma laidlawii* strain A colonies, some of which had already developed the peripheral zone (Pl. 1, fig. 2). The L-form grew faster than the Mycoplasma and almost all colonies developed into the 'fried egg' type after 48 hr. incubation (Pl. 1, fig. 5). The peripheral zone of the colonies, located on the agar surface, was easily removed by scraping. Mature colonies of both organisms may be seen in Pl. 1, figs. 3, 6. These colonies had big peripheral zones.

The development of colonies was also followed quantitatively by measuring the diameter of the colonies and their central zones (Fig. 1). The central zone was the first to develop, followed by the formation of the peripheral zone later on. The central zone grew only a little after 48-hr. incubation, whereas the peripheral zone continued to grow, until it occupied the greater part of the colony after incubation for 96 hr.

Vertical sections of mature colonies

All Mycoplasma and L-form strains used in this investigation were examined. The colony sections showed the same basic structure in all organisms: a hemispherical central zone which is embedded in the agar and a thin peripheral zone which spreads over the agar surface (Pl. 2, figs. 7–10). The central zone was a firm and elastic structure in comparison with the very soft peripheral zone.

Penetration of Mycoplasma and L-forms into the agar and formation of the central zone

In order to understand the mechanism of formation of Mycoplasma and L-form colonies we thought it worth while to investigate why the growth of these organisms begins in the agar. Experiments to this purpose showed that the organisms penetrated into the agar medium before multiplication. As may be seen from

Table 1, immediately after the drops had dried about 50 % of the Mycoplasma organisms could be transferred to the agar slices. Further incubation gradually decreased the number of organisms that could be transferred and at 6–8 hr. after inoculation almost all organisms remained on the agar plate.

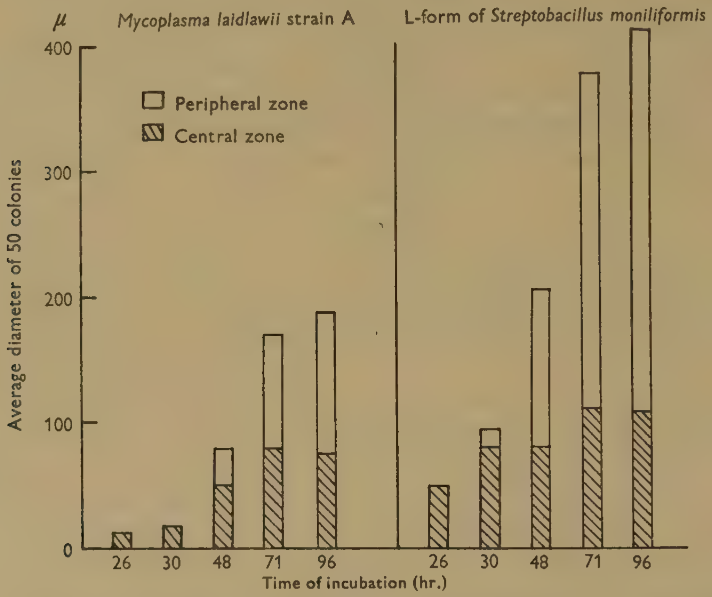

Fig. 1. The development of colonies of *Mycoplasma laidlawii* strain A and the L-form of *Streptobacillus moniliformis* on solid Edward medium.

The sum of the number of colonies which developed on the drop area on the plate and the corresponding agar slice remained constant within the first 8 hr. of experiment, indicating that no mortality or multiplication took place during this period. After 24-hr. incubation the number of colonies which appeared on the agar slices increased and became equal to the number of colonies on the corresponding drop areas. However, in most experiments the growth was confluent. This was due to the smear of the surface peripheral zones which began to appear at this time (Pl. 1, figs. 2, 4).

The four Mycoplasma strains tested behaved similarly; almost all organisms penetrated into the agar within 6–8 hr. after inoculation. The L-forms of *Proteus* sp. and *Streptobacillus moniliformis* differed in this respect since the number of organisms which could be transferred to the agar slice did not change significantly during the first 8 hr. of the experiment.

For comparison the same experiment was repeated with *Staphylococcus aureus* and *Streptococcus pyogenes*. More than 50 % of the organisms inoculated could be transferred to the agar slices after 3-hr. incubation, indicating that these organisms did not penetrate into the agar.

Mycoplasma penetration into the agar did not occur when the surface of the agar was moist. Moist Edward agar plates were prepared by pouring different amounts of sterile distilled water on the agar surface. With *Mycoplasma laidlawii* strain A as

Table 1. *The penetration of Mycoplasma and L-forms of bacteria into agar*

All experiments were done in duplicate and some in triplicate. The table gives typical results of some of the experiments. For technical details see text.

a = number of colonies found on the drop area on the plate.
b = number of colonies found on the corresponding agar slice.

Hr. after inoculation	*Mycoplasma laidlawii* strain A		*Mycoplasma laidlawii* strain B		*Mycoplasma mycoides* var. *capri*		*Mycoplasma bovigenitalium*		L-form of *Proteus*		L-form of *Streptobacillus moniliformis*		*Staphylococcus aureus**		*Streptococcus pyogenes**	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
0	110	80	55	44	167	160	55	45	152	215	83	78	6	16	29	63
1	136	70	45	48	166	102	68	26	201	152	70	71	11	16	25	68
3	140	45	80	32	210	55	59	30	244	154	125	105	10	14	120	260
6	170	33	103	12	250	45	58	25	216	162	129	128	—	—	**	**
8	200	30	90	8	270	18	70	35	190	138	144	79	**	**	**	**
24	235	225	**	**	**	**	**	**	**	**	**	**	—	—	—	—

*, in this experiment thallium acetate and penicillin were omitted from the Edward medium.
**, confluent growth.

inoculum, growth was inspected after incubation at 37° for 3 days in a moist atmosphere. The colonies which appeared on the surface of the moist plates were large, flat, and had either minute central zones or none at all (Pl. 2, figs. 11, 12).

Suspensions of Mycoplasma or L-forms were mixed with melted Edward agar at 45° and poured into plates. Most colonies developed deep inside the agar, forming ball-like structures, in contrast to the lens-like deep agar colonies of Eubacteria such as *Staphylococcus aureus* (Pl. 3, figs. 13–15).

Factors influencing the form and dimensions of the peripheral zone

It was assumed that the peripheral zone of Mycoplasma and L-form colonies develop in the thin free water film which is present on the agar surface (Knaysi, 1951). This view was supported by the demonstration of the spreading of peripheral zones along cotton-wool fibres placed on the agar (Pl. 3, figs. 16, 17).

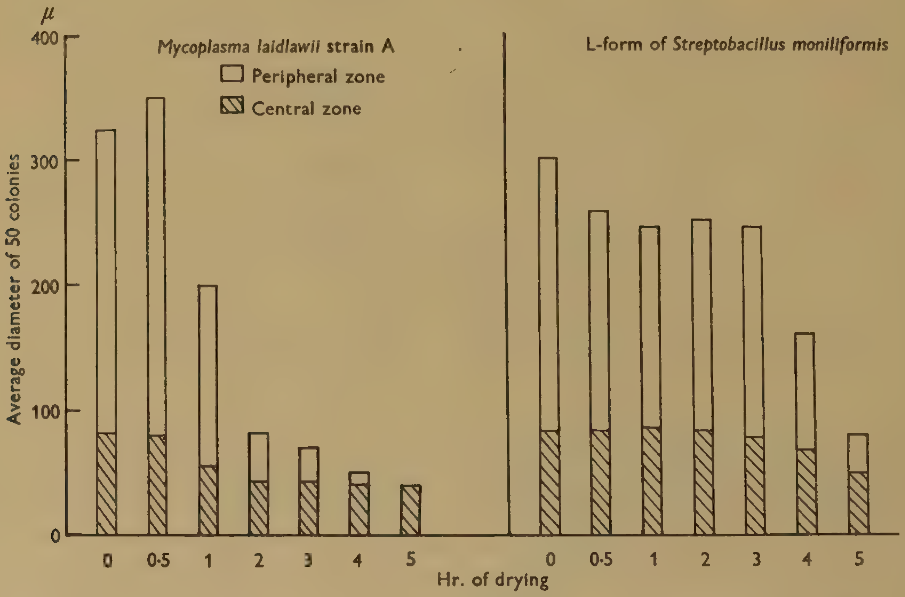

Fig. 2. The effect of pre-drying the Edward medium on the morphology of colonies of *Mycoplasma laidlawii* strain A and the L-form of *Streptobacillus moniliformis*.

The need of a humid atmosphere for optimal growth of Mycoplasma and L-forms has been stressed by many authors (Edward, 1954; Klieneberger-Nobel, 1954). Drying of the plates and subsequent incubation in a dry atmosphere decreases the free water film on the agar surface, possibly affecting the formation of the peripheral zones. To test this assumption, solid Edward medium in Petri dishes was dried for different periods of time by opening the plates in a 37° incubator. After the inoculation with suspensions of *Mycoplasma laidlawii* strain A and the L-form of *Streptobacillus moniliformis* the plates were placed in tins without moist cotton wool and incubated at 37° for 3 days. The dimensions of the peripheral zones were found to be markedly decreased in the case of the dried plates (Fig. 2). Drying for 5 hr. caused complete disappearance of the peripheral zones in all colonies of *M. laidlawii* strain A and in almost all colonies of the L-form of *S. moniliformis*. The rate of reduction of the central zone diameter due to drying of the medium was much smaller than that of the peripheral zone diameter.

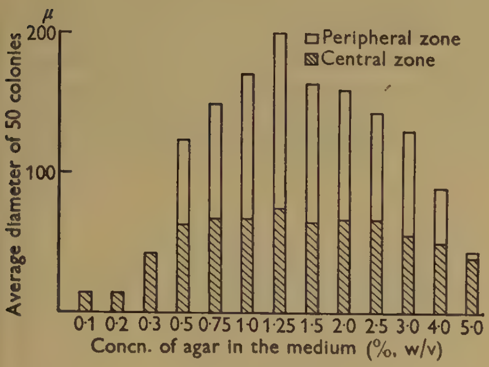

Fig. 3

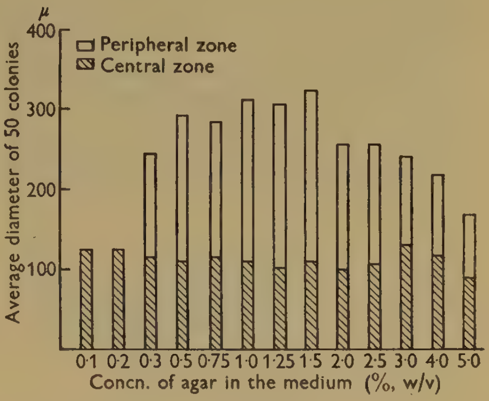

Fig. 4

Fig. 3. The effect of agar concentration in Edward medium on the morphology of *Mycoplasma laidlawii* strain A colonies.

Fig. 4. The effect of agar concentration in Edward medium on the morphology of colonies of the L-form of *Streptobacillus moniliformis*.

The effect of agar concentration on the morphology of the colonies

The effect of changes in the agar concentration in Edward medium on the morphology of colonies of *Mycoplasma laidlawii* strain A and the L-form of *Streptobacillus moniliformis* is illustrated in Figs. 3 and 4. With low agar concentrations (0·1–0·3 %, w/v), the medium was semisolid and *M. laidlawii* strain A grew in small irregular ball-like colonies at various depths of the medium. A hazy surface growth was also present. Typical colonies, consisting of both central and peripheral zones, appeared at a concentration of 0·5 % (w/v). Increasing the concentration of the agar to 1·25 % (w/v) increased the diameter of the colonies, mainly that of the peripheral zones. Further increase in agar concentration caused a gradual decrease of the colony diameter, affecting primarily the peripheral zone. At a concentration of 5 % (w/v) agar, almost all colonies were without peripheral zones. Similar results were obtained with the L-form of *S. moniliformis* (Fig. 4). The diameter of the central zone of the L-form colonies did not change significantly by changing the agar concentration of the medium. The optimal agar concentration for the development of the peripheral zone was about 1·5 % (w/v); higher concentrations inhibited its formation. A hazy surface growth was observed with low agar concentrations (0·1–0·2 %, w/v).

Morphology of Mycoplasma colonies on solid media other than agar

Mycoplasma laidlawii strains A and B and *M. mycoides* var. *capri* were grown on Edward medium solidified with 12 % (w/v) gelatin instead of agar. The plates were incubated for 3 days at 25°. Growth appeared mostly in the shape of ball-like colonies at various depths of the medium. Some colonies near the surface also developed peripheral zones. Liquefaction of the gelatin by the organisms after further incubation masked this typical colony morphology.

Mycoplasma laidlawii strain A gave rise to colonies composed of central and peripheral zones when grown on the cellulose pellicle of *Acetobacter xylinum*. Many colonies, however, had no peripheral zones since they had developed deep inside the pellicle, resembling the deep agar colonies of Mycoplasma (Pl. 3, fig. 13).

Mycoplasma laidlawii strain A gave flat surface colonies without central zones when grown on coagulated serum medium. The same type of surface colonies, not penetrating into the medium, appeared when this organism was grown on the surface of a cellophan dialysis membrane placed on solid Edward medium.

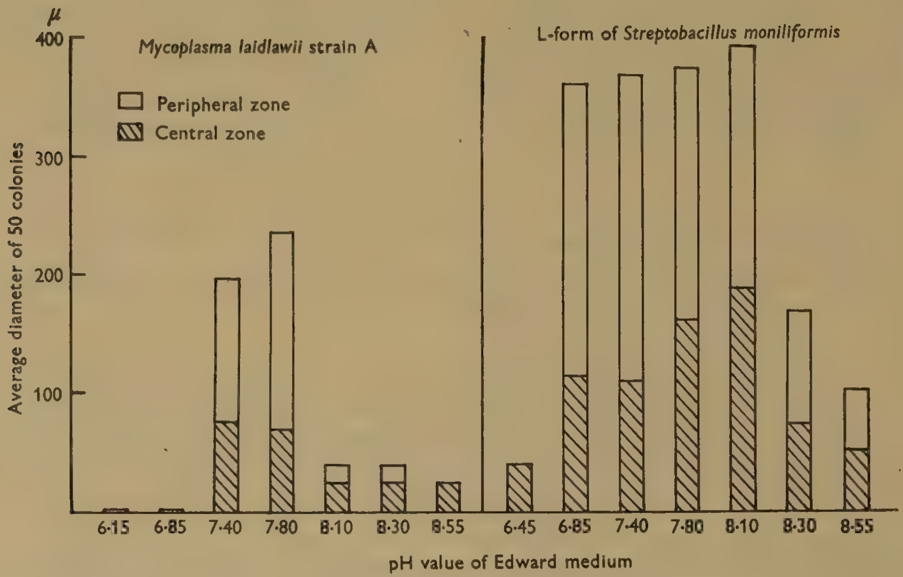

Fig. 5. The effect of pH value on the growth of *Mycoplasma laidlawii* strain A and the L-form of *Streptobacillus moniliformis* in Edward medium.

The effect of inorganic salts and hydrogen ion concentration on the morphology of Mycoplasma and L-form colonies

Sodium chloride or $Li_2SO_4.H_2O$ were incorporated into solid Edward medium in concentrations of 0·5, 1·0, 2·0 and 3 % (w/v). The plates were inoculated with *Mycoplasma laidlawii* strain A and the L-form of *Streptobacillus moniliformis* and the growth inspected after 3 days of incubation at 37°. Normal growth of *M. laidlawii* strain A occurred at concentrations 0·5 and 1·0 % (w/v) with both salts. At a salt concentration of 2·0 % (w/v) a partial inhibition of growth was noted; the colonies were much smaller and many lacking in peripheral zones. With 3·0 % (w/v) of $Li_2SO_4.H_2O$ none of the colonies had peripheral zones, while NaCl at a similar concentration inhibited growth completely. The growth of the L-form of *S. moniliformis* was completely inhibited with 2·0 % and 3·0 % concentration of both salts. At a salt concentration of 1 % (w/v) most colonies appeared with central zones only, while 0·5 % (w/v) of NaCl or $Li_2SO_4.H_2O$ gave normal colonies.

The pH value of the medium has a marked influence on the growth of Mycoplasma organisms (Edward, 1954; Peoples, Morton & Feo, 1957). The growth and colony morphology of *Mycoplasma laidlawii* strain A and the L-form of *Streptobacillus moniliformis* were tested at various pH values of Edward medium (Fig. 5). *M. laidlawii* strain A did not grow at pH values below 6·85; optimal growth occurred at pH 7·8. Increasing the pH above 8·0 decreased growth, causing most colonies to appear without peripheral zones. At pH 8·55 peripheral zones were absent from all colonies. The L-form of *S. moniliformis* was less sensitive to pH changes than the Mycoplasma, but inhibition of growth also occurred at low or high pH values, accompanied by the appearance of small colonies without peripheral zones.

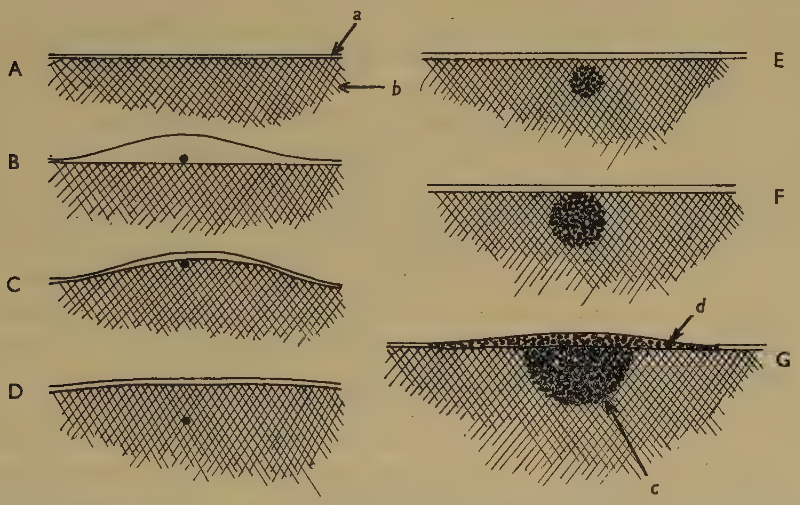

Fig. 6. Diagram to illustrate the development of Mycoplasma and L-form colonies. (A) Vertical section through the agar before inoculation: *a*, free water film; *b*, network of agar fibrils. (B) A drop containing a viable particle of Mycoplasma or L-form is placed on the agar. (C) C. 15 min. after inoculation: the drop is absorbed by the agar forming a slight swelling. (D) C. 3–6 hr. after inoculation: the viable particle has penetrated into agar. (E) C. 18 hr. after inoculation: a small ball-like colony has been formed inside the agar. (F) C. 24 hr. after inoculation: the ball-like colony approaches the agar surface. (G) C. 24–48 hr. after inoculation: the growth spreads into the free water film forming the peripheral zone: *c*, central zone; *d*, peripheral zone.

DISCUSSION

On the basis of our findings and those already reported by others, we are able to propose a possible mechanism for the formation of Mycoplasma and L-form colonies (shown schematically in Fig. 6). Under normal conditions the multiplication of these organisms begins inside the agar. The inoculated organisms are drawn into the agar gel together with the water surrounding them. The dried agar surface has strong imbibition properties, and water is absorbed quickly by capillary forces (Porter, 1946). This can be demonstrated by placing a drop of water on the surface of a dry agar plate; the drop is absorbed within a few minutes, resulting in a slight swelling of the agar at the drop area. When the agar surface is moist the absorption of additional water is decreased or even abolished. Therefore penetration of the organisms is very slight, or absent altogether, resulting in the formation of surface colonies with small or no central zones (Pl. 2, figs. 11, 13). Hence, the forces responsible for drawing the micro-organisms into the agar are the capillary forces of the agar gel. The determinant factors of the actual penetration, however, are the very small dimensions and the plastic nature of the Mycoplasma and L-forms, allowing these organisms to enter between the agar fibrils and to move in the interstices of the fibrillar network of the agar gel.

Klieneberger-Nobel (1956) showed by filtration experiments and electron microscopy that L-form cultures contain viable particles of various sizes, the smallest of which have a diameter of about 300 mμ and occur only in small numbers. In contrast, cultures of Mycoplasma are composed mainly of very small viable particles, which have an average diameter of about 100–150 mμ. These differences between L-forms and Mycoplasma might explain the results shown in Table 1. Most viable particles of Mycoplasma penetrated into the agar, whereas only a part of the viable particles of the L-forms showed this ability.

The absence of motility in Mycoplasma and L-forms (Klieneberger-Nobel, 1954, 1960) excludes the possibility of active penetration of the organisms into the agar. Gravity forces do not appear to play any role in the penetration since this process is not affected by incubating the inoculated plates upside down. Actinomycetes are known to penetrate into the agar by their filamentous growth (Knaysi, 1951). Some Mycoplasma species also produce filaments and Freundt (1960) has expressed the opinion that this is a common feature of all Mycoplasma species. The possibility that the Mycoplasma penetrate into the agar through the formation of filaments is, however, disproved by the fact that penetration is accomplished within a few hours after inoculation, before the occurrence of any growth and multiplication (Table 1).

It is further shown by our results that the multiplication of the Mycoplasma and L-forms begins inside the agar at various distances from the surface, depending on the initial depth of penetration of the viable particle giving rise to the colony. During the process of multiplication the organisms do not push aside the agar, because they can penetrate and occupy the interstices between the agar fibrils. This is demonstrated by the finding that the growth of Mycoplasma and L-forms in the agar results in the formation of firm ball-like colonies instead of the lens-shaped colonies formed by the usual Eubacteria inside the agar (Pl. 3, figs. 13–15). The formation of lens-shaped deep agar colonies is explained by the inability of the usual bacteria to penetrate into the interfibrillar spaces, therefore causing the agar to split during the multiplication; the colony then spreads along this split (Knaysi, 1951). Staphylococci and streptococci do not penetrate into the agar, as shown in Table 1. It may be concluded that the central zone with Mycoplasma is a ball composed of a network of agar fibrils, with the soft micro-organisms occupying the interstices. The agar fibrils are responsible for the firmness of the central zone.

The ball-like growth inside the agar continues to grow and expand in all directions until it reaches the agar surface which is usually covered by a thin film of free water (Knaysi, 1951). At this stage the organisms spill into this free water film, forming the peripheral zone. The surface tension of the water is responsible for the circular shape of the peripheral zone. The latter is very soft because it does not contain agar, in contrast to the firm central zone. Cotton-wool fibres on the agar cause the peripheral zones of adjacent colonies to lose their round form and to spread along the fibres due to changes in the surface tension of the water surrounding the fibres. This is further evidence for the location of the peripheral zone in the free water film of the agar.

The dimensions of the peripheral zone are determined by the thickness of the free water film. The thinner the film, the smaller will be the peripheral zone, and the colony as a whole. This explains, at least partly, the importance of a fresh medium and a humid atmosphere for optimal growth of Mycoplasma (Edward, 1954).

It is interesting to note that the large bodies, typical of L-forms and Mycoplasma, appear only at the peripheral zone of the colony (Liebermeister, 1960). This phenomenon might be explained by remembering that inside the agar the organisms are pressed together by the agar fibrils, whereas in the peripheral zone they are free to expand either by growth of the cytoplasm, or by swelling due to low osmotic pressure of the free water film.

The necessity of a fibrillar structure of the medium for the formation of the central zone was demonstrated with the cellulose pellicle of *Acetobacter xylinum*, known to be composed of a network of cellulose fibrils (Mühlethaler, 1949). The typical morphology of Mycoplasma and L-form colonies on gelatin medium apparently depends also on the fibrillar nature of this gel. Hayflick & Stinebring (1960) reported the formation of typical Mycoplasma colonies on the fibrillar plasma clot. On the other hand coagulated serum, which is not fibrillar, does not allow penetration of the organisms and the typical 'fried egg' colony does not appear with this medium (Dienes, 1960).

Apparently the wider interstices between the cellulose and gelatin fibrils, compared to agar at a concentration of 1·25 % (w/v), enable the organisms to penetrate deeper into the cellulose and gelatin media. Thus, the inside growth frequently cannot reach the surface and grows into a ball-like colony inside the medium. The same may happen when the concentration of the agar in Edward medium is decreased. The wider interstices between the fibrils at low agar concentrations explain also the smallness and the irregular boundaries of the central zones observed at these concentrations. The larger viable particles of the L-forms (Klieneberger-Nobel, 1956) apparently do not penetrate quite so deeply into the agar (Table 1), thus explaining the development of large central zones and the appearance of peripheral zones at lower agar concentrations than with the Mycoplasma. The decrease of the peripheral zone dimensions observed at high agar concentrations might be explained by the diminution of the free water film under these conditions (Knaysi, 1951). Increasing the agar concentration also causes decrease of the central zone diameter, apparently due to the inhibition of penetration of the organisms into the medium.

It is noteworthy that when the medium becomes unfavourable for growth, as in the presence of high concentrations of inorganic salts or unsuitable hydrogen ion concentrations, the peripheral zone is the first to disappear, presumably because the initial ball-like growth inside the agar is not big enough to reach the surface and form the peripheral zone. This observation might explain the formation of the T-colonies of Mycoplasma (Shepard, 1960), which are composed apparently only of central zones embedded in the agar (Hayflick & Stinebring, 1960). This is due to the unsuitability of the medium to support optimal growth of these exacting Mycoplasma strains. This was also our experience while testing defined media for Mycoplasma. Poor media only gave rise to this type of agar-embedded colonies without peripheral zones.

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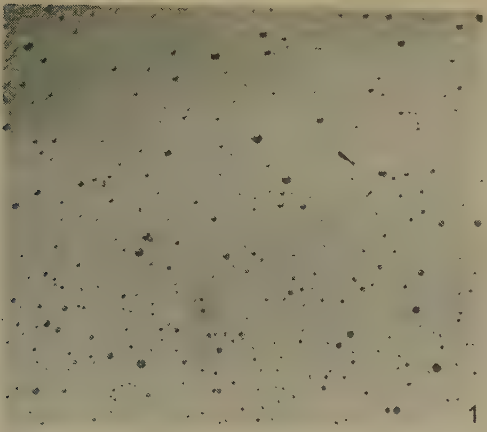
EXPLANATION OF PLATES

Plate 1

Figs. 1–3. The development of *Mycoplasma laidlawii* strain A colonies on Edward medium after 24, 48 and 96 hr. incubation at 37°. × 50.

Figs. 4–6. The development of the L-form of *Streptobacillus moniliformis* colonies. Medium and incubation periods as above. × 50.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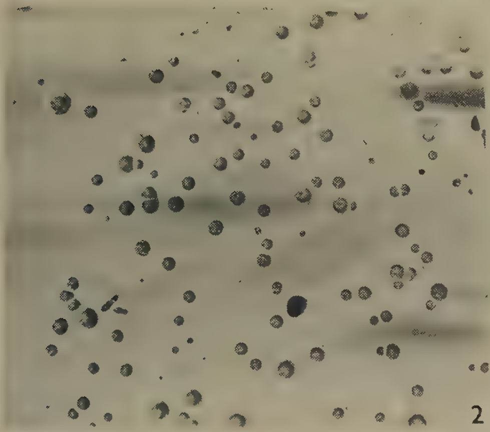
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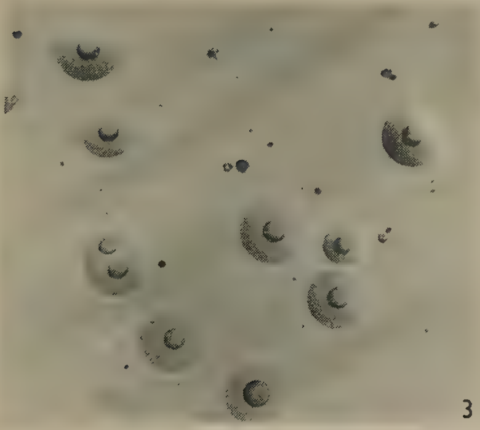
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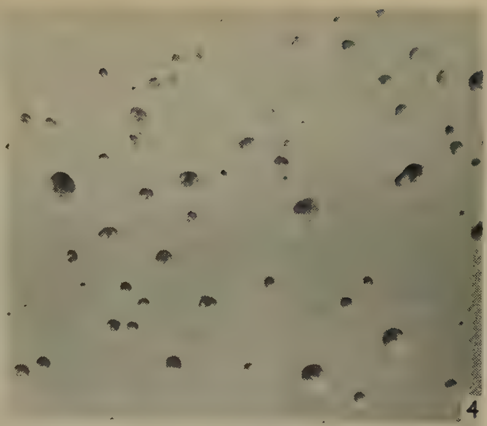
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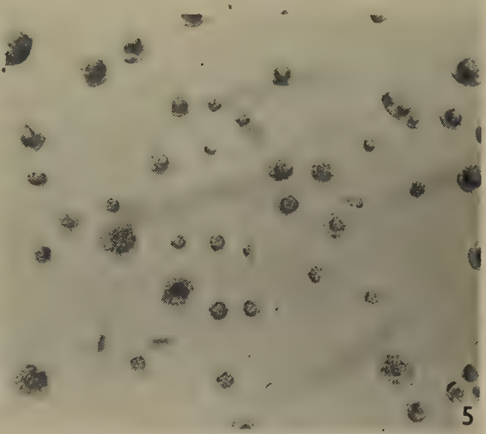
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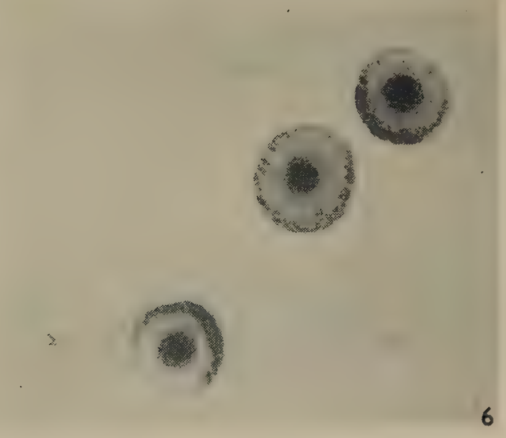
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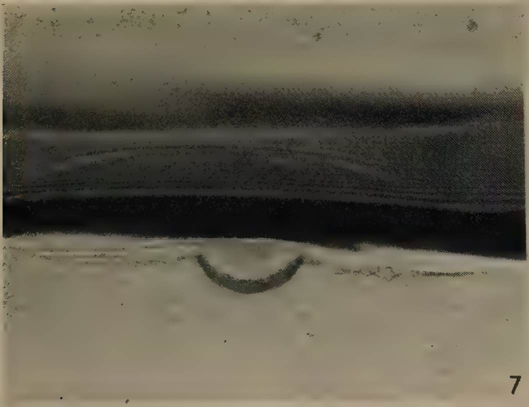
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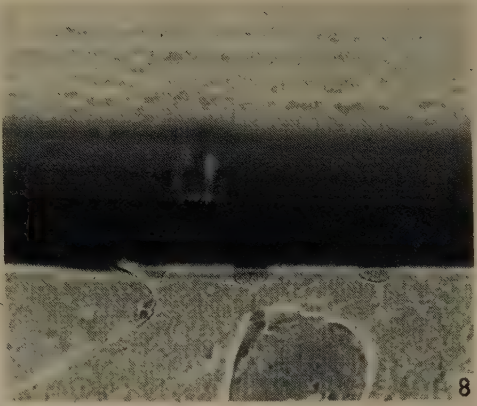
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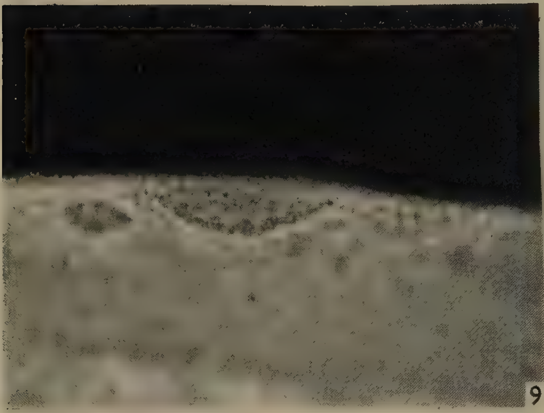
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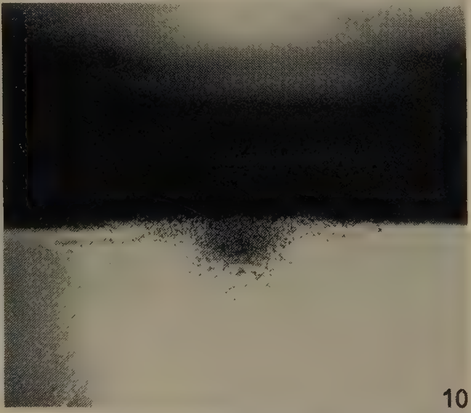
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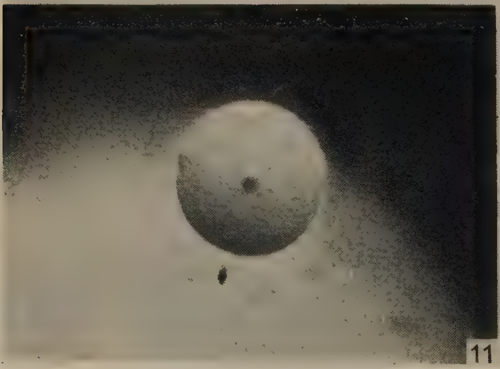
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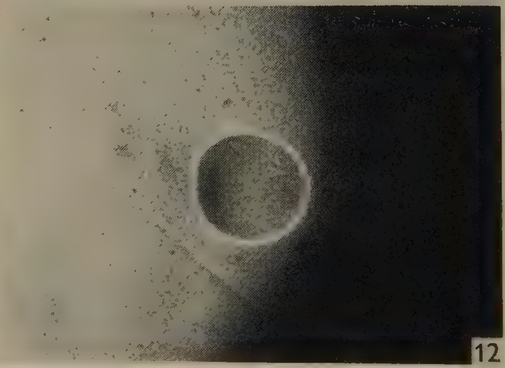
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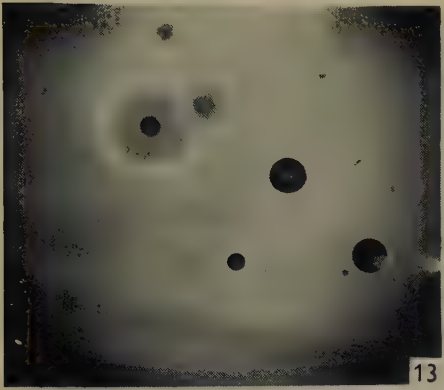
S. RAZIN AND O. OLIVER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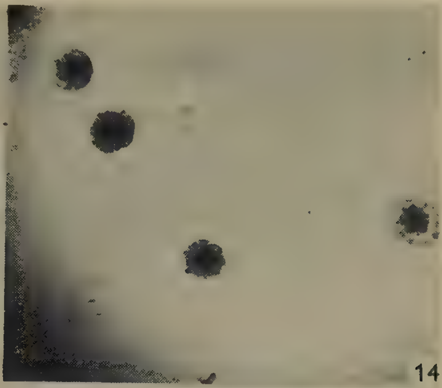
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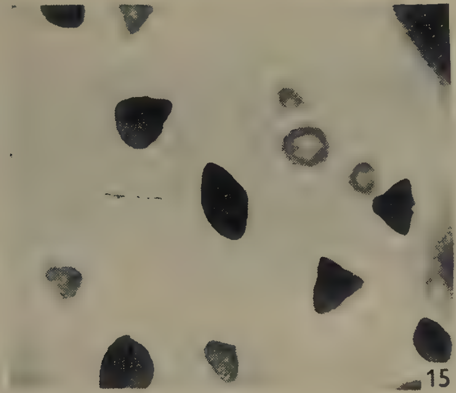
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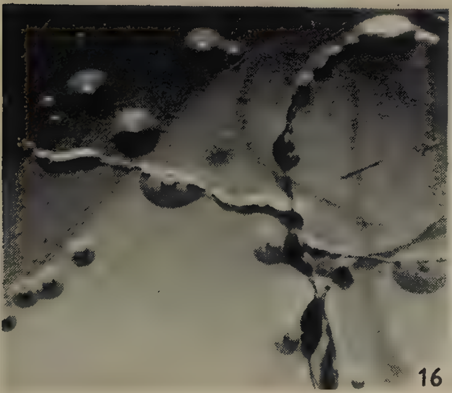
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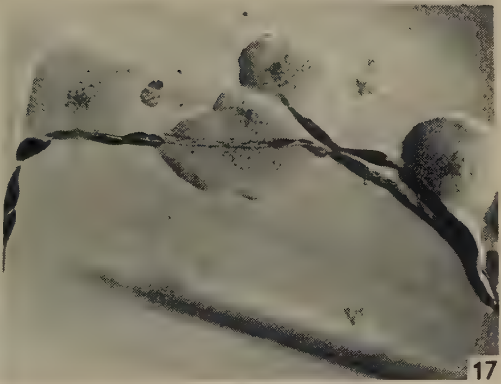
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S. RAZIN AND O. OLIVER

PLATE 2

Figs. 7–10. Vertical sections of colonies.

Fig. 7. *Mycoplasma laidlawii* strain A. ×150.

Fig. 8. *Mycoplasma mycoides* var. *capri.* ×150

Fig. 9. *Mycoplasma gallisepticum.* ×600.

Fig. 10. L-form of *Proteus.* ×150.

Figs. 11, 12. Colonies of *Mycoplasma laidlawii* strain A grown on moist Edward agar. ×50.

PLATE 3

Fig. 13. Deep Edward agar colonies of *Mycoplasma laidlawii* strain A. Including one surface colony with a peripheral zone. ×50.

Fig. 14. Deep Edward agar colonies of the L-form of *Streptobacillus moniliformis.* One small surface colony is seen at the right end of the photograph. ×50.

Fig. 15. Deep Edward agar colonies of *Staphylococcus aureus.* ×50.

Fig. 16. *Mycoplasma laidlawii* strain A colonies touching cotton-wool fibres on the surface of Edward medium. ×50.

Fig. 17. L-form of *Streptobacillus moniliformis* colonies touching cotton-wool fibres on the surface of Edward medium. ×50.

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The Influence of Hydrogen Ion Concentration and 2:4-Dinitrophenol on Orthophosphate Accumulation in *Tetrahymena pyriformis*

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(*Received* 9 *August* 1960)

SUMMARY

The relationship of the pH value of the suspension medium to inorganic phosphate accumulation by *Tetrahymena pyriformis*, strain W, in the presence and absence of 2:4-dinitrophenol was studied. Investigations were conducted to ascertain whether variations of the conditions of culture influenced phosphate accumulation. The pH value of the suspension medium had an influence on the amount of radiophosphate accumulation. The pH optimum, under the conditions used, was 6·5. The pH value of the culture medium, in the range tested, had little or no influence on the pH optimum observed in the suspensions, although differences in total phosphate accumulation were noted. Approximately 70 % of the orthophosphate accumulated by the organisms was in the form of inorganic phosphate, which leads to the postulate of an active transport mechanism for phosphate accumulation in Tetrahymena. 2:4-Dinitrophenol was most effective in lowering radiophosphate accumulation in an acidic medium, and became less effective with increasing alkalinity. The results can be explained on the basis of the penetration of only the undissociated molecule into the organisms. It is suggested that 2:4-dinitrophenol interferes with the transport mechanism involved in phosphate entry.

INTRODUCTION

The growth of *Tetrahymena pyriformis* has been reported to be attenuated in the presence of 2:4-dinitrophenol (DNP). This growth inhibition is annulled by the addition of stigmasterol or related sterols to the medium (Conner, 1957). Numerous investigations concerning the effects of DNP on a variety of biological systems indicate that metabolic activities which require a constant supply of energy are adversely affected. DNP retards or attenuates active transport of cations in yeast (Conway, 1955), protein synthesis in normal and malignant rat livers (Frantz, Zamecnek, Reese & Stephenson, 1948), cleavage in Arbacia eggs (Clowes & Krahl, 1936), photosynthesis and photo-reduction in *Scenedesmus* (Gaffron, 1942), and sodium ion extrusion from the squid axon (Caldwell, 1957). The widespread effects of DNP on cellular activities can be explained in terms of an adverse effect on the enzymic reactions which lead to the production of energy-rich phosphate groupings such as adenosine triphosphate (ATP; Loomis & Lipmann, 1948). The formation of such groupings involves the esterification of orthophosphate (Hunter, 1951), and if such reactions were blocked, it might be expected that less orthophosphate would tend to enter the cell, either because of a lowered orthophosphate demand or a lowered availablity of energy necessary for accumulation. In some cases it has been shown that the extent to which DNP influences metabolic processes is related to the pH value of the solution used for suspending the biological material (Caldwell, 1957; Simon, 1953; Hamburger & Zeuthen, 1957). This indicates the necessity of controlling the pH value when evaluating the effect of a given concentration of DNP on a biological system. The extent to which DNP affects phosphate metabolism may depend on the intracellular concentration of the substituted phenol, which in turn might depend on the pH value of the suspension medium. Also, the influence of the pH value on the amount of phosphate accumulated is an important consideration.

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This paper deals with the influence of the pH value of the medium on DNP inhibition of growth of Tetrahymena, on orthophosphate accumulation in the presence and in the absence of DNP, and with the relationship of orthophosphate accumulation to the pH of the medium in which the organisms were cultured. The distribution of the accumulated phosphate between organic and inorganic forms was also determined.

METHODS

The organisms, *Tetrahymena pyriformis*, strain W, were cultured under axenic conditions in a medium composed of (%, w/v): 2, proteose peptone (Difco); 0·1, yeast extract (Difco); 1, glucose; 0·2, sodium acetate; 0·15 tris buffer (2-amino-2-hydroxymethyl propane-1:3 diol methane; Sigma 121). In all cases, glucose was autoclaved separately and then added to the medium. For the various experiments the media were adjusted at the time of inoculation to pH values of 5·5, 6·5, 7·0 and 7·5 as determined by the Beckman pH meter. The organisms were incubated for 72 hr. in the dark at 25° ± 2°, harvested by centrifugation (200 *g*), washed three times with distilled water, and suspended in water to give an optical density reading of 0·5 on a Lumetron Colorimeter (Model 401 A) equipped with a 650 mμ filter. The packed organisms of 1 ml. of this suspension had a volume of 0·015 ml.

The phosphate accumulation experiments were carried out in flasks containing the following: 0·012 M-tris buffer, 0·01 M-sodium acetate, $1{\cdot}5 \times 10^{-8}$ M-radiophosphate (0·008 μc./ml. final concentration), 1×10^{-4} M-DNP when appropriate, and 1 ml. of Tetrahymena suspension. The final volume in all flasks was 10 ml. In the experiments involving fractionation of the phosphates, twice the number of organisms was added and a final volume of 20 ml. used.

The organisms were allowed to stand for 1 hr. in the presence of tris buffer; then sodium acetate and DNP were added to the appropriate flasks. Fifteen minutes later all flasks were charged with radiophosphate. After incubation for 2 hr. with radiophosphate the organisms were removed by filtration or centrifugation.

In the experiments involving the fractionation of the accumulated phosphate into acid-insoluble, acid-soluble organic and inorganic portions the following procedure was used. After the 2 hr. incubation period with the radiophosphate, 5 ml. of suspension were filtered to remove the organisms. A 10 ml. portion of the suspension was added to an equal volume of 10 % (w/v) trichloroacetic acid (TCA), the mixture allowed to stand for 1 hr., followed by centrifugation. To a 10 ml. portion of the TCA supernatant fluid the following reagents were added in the order given: 1·0 ml. KH_2PO_4 (0·2 %, w/v); 2·0 ml. $MgSO_4.7H_2O$ (15 %, w/v); 1·5 ml. ammonia (sp.gr. 0·880). The contents were mixed thoroughly, covered, and allowed to stand overnight. The following morning the precipitate of magnesium ammonium phosphate was sedimented by centrifugation, washed with dilute ammonia (sp.gr. 0·880, diluted 1/10) and resedimented. The supernatant fluid was discarded, the tube drained by inversion and the precipitate dissolved in 1 ml. concentrated HCl, and the volume adjusted to 5 ml. with water. The temperature of all solutions was maintained at 5° during the fractionation procedure.

The radioactivity of three 1 ml. samples was determined by using a thin window Geiger-Muller tube and scaler. Constant geometry was maintained and all counts were corrected for background. The solutions on which counts were taken to assess radioactivity are designated as follows: measure of initial radioactivity in the suspension medium, T_0; suspension medium after removal of organisms, T_2; TCA supernatant fluid, TCA; inorganic phosphate, P_i. The P_i counts were taken at 103 % to compensate for the decay which occurred over the period of about 18 hr.

Direct counts were not made of the TCA-soluble organic or the TCA-insoluble fractions because quantitative recovery could not be achieved. To determine the values of these fractions and to correct for dilution the following calculations were made: total radiophosphate removed from the suspension medium (accumulated phosphate), $T_0 - T_2$; TCA insoluble, $T_0 - 2\text{TCA}$; TCA soluble organic, $2(\text{TCA} - P_i)$; inorganic phosphate, $2P_i - T_2$. Variance was computed for each set of original counts, corrected for background:

$$\frac{\Sigma(X-\bar{X})^2}{N-1},$$

where X is count value, $\bar{X}$ is mean value, N is number of samples counted.

Standard deviation of the calculated count values was determined as:

$$\sqrt{\frac{s_x^2+s_y^2}{N-1}},$$

where s_x^2 is variance of the 1st term, s_y^2 is variance of the 2nd term. (When x or y was increased by a factor of two to correct for dilution, s^2 was doubled for determination of standard deviation.)

Percentage deviation was found by taking the extreme values as given by standard deviations:

$$\frac{X+s_x}{Y-s_y}-\frac{X}{Y},$$

where X is the calculated count value for a given fraction, Y is either T_2 counts or accumulated phosphate in the fractionation experiments.

RESULTS

The influence of 2:4-*dinitrophenol and* pH *value of the culture medium on growth*

The results of the growth experiment (see Fig. 1) indicate that the rate of multiplication of the organisms decreased with increasing DNP concentration. At a given concentration of DNP, the rate of growth decreased with increasing acidity. In the absence of DNP, no pH effect was seen in the final concentrations of organisms, confirming the findings of Kidder (1941).

Tetrahymena pyriformis used for the phosphate accumulation studies was grown in proteose-peptone medium adjusted to the desired pH value. The pH values of the media after growth for 72 hr. are given in Table 1. In all cases the pH value of the media moved towards neutrality. Orthophosphate accumulation was similar in organisms which had been grown in media initially at pH 5·5 and 6·5, but was somewhat lower in the case of organisms grown in media initially at pH 7·5. In fifteen experiments there was no exception to the pattern of radiophosphate accumulation, although differences in the absolute amounts of orthophosphate accumulated were noted from experiment to experiment. In general, a shift in the pH value of the suspension medium towards neutrality took place during the experimental period. However, in spite of this tendency to approach neutrality, the initial pH value of the suspension medium had an influence on the degree of orthophosphate accumulation. A pH optimum of 6·5 was found regardless of the conditions of culture used.

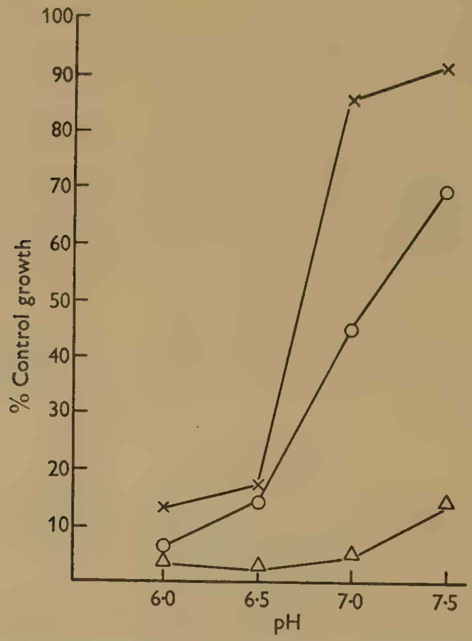

Fig. 1. Effect of 2:4-dinitrophenol (DNP) in media at different pH values on the growth of Tetrahymena. (×) 5×10^{-5}M, (O) 1×10^{-4}M, (Δ) 2×10^{-4}M-DNP. The media and conditions were as reported previously (Conner, 1959).

Orthophosphate accumulation by *Tetrahymena pyriformis* was inhibited by DNP. The maximal inhibition for a given concentration of DNP occurred when the suspension medium was acidic (see Fig. 3). In the presence of DNP, the pH optimum at 6·5 was abolished.

Since the pH value of the suspension medium had an effect on phosphate accumulation in absence of DNP, the data require clarification. When the amount of radio-

Table 1. pH *Value of culture media before and after growth of Tetrahymena*

The protozoans were maintained at 25° ± 2 for a period of 72 hr.

Initial pH of culture media	pH after growth for 72 hr.
5·5	6·3–6·4
6·5	6·7–6·8
7·5	7·2–7·3

phosphate accumulated at each pH value in the presence of DNP is compared with a control at that pH value, i.e. % DNP accumulation/% control accumulation, a better description of the influence of the pH of the suspension medium with regard to DNP inhibition is obtained (see Table 2). The distribution of accumulated phosphate (taken as 100 %) was TCA-insoluble 19·8 % ± 3·8, organic TCA-soluble 11·3 % ± 3·7, inorganic phosphate 68·9 % ± 3·5. In these experiments the organisms were grown at pH 6·5 and the pH value of the suspension medium was 7·0. The phosphate removed from the suspension medium by the Tetrahymena was 75 % ± 5 (seven experiments). In a few experiments the organisms were incubated in a suspension medium at pH of 6·5 or 7·5. There was no significant variation in the distribution of phosphate under these conditions.

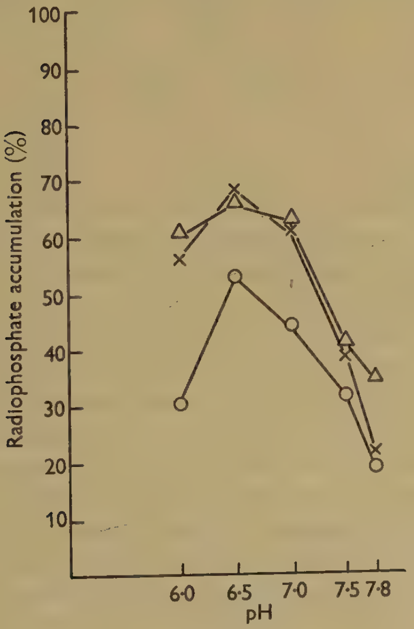

Fig. 2

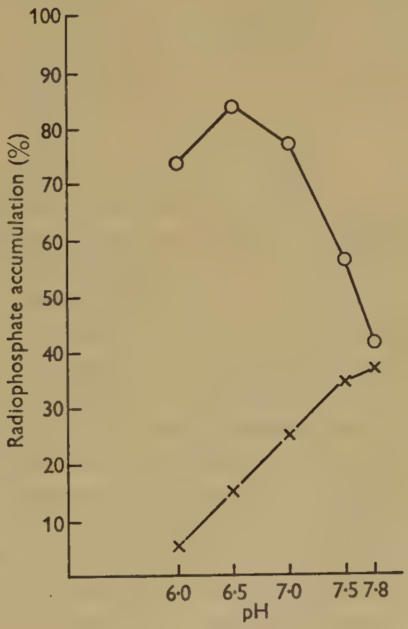

Fig. 3

Fig. 2. Influence of pH value of the suspension medium on radiophosphate accumulation by Tetrahymena grown in proteose peptone media of pH 5·5 (×), 6·5 (Δ) and 7·5 (○). Each point represents the mean % accumulation for five experiments.

Fig. 3. The influence of the pH value of the suspension medium on radiophosphate accumulation by Tetrahymena in the presence (×) or absence (○) of 1×10^{-4}M-DNP. The organisms were grown in the proteose peptone medium (pH 6·5).

Table 2. *Data from representative experiments on accumulation of radiophosphate by* Tetrahymena *in the presence and absence of* 10^{-4}M DNP *under various conditions of* pH *of culture medium and suspension medium*

Organisms grown in medium at pH 5·5 (see Table 1)

Initial pH value of suspension medium (T_0)	6·0	6·5	7·0	7·5	7·8
Terminal pH value of control (T_2)	6·4	6·75	6·95	7·4	7·7
Terminal pH value in presence of DNP	6·55	6·8	6·95	7·4	7·7
% Accumulation control (no DNP)*	47·4	70·8	61·6	34·8	30·6
% Accumulation in presence of DNP*	1·4	11·2	35·0	39·1	27·9
% DNP/% Control	3	16	57	112	91

Organisms grown in medium at pH 6·5

Initial pH value of suspension medium (T_0)	6·0	6·5	7·0	7·5	7·8
Terminal pH value of control (T_2)	6·6	6·8	6·9	7·3	7·6
Terminal pH value in presence of DNP	6·4	6·7	6·9	7·2	7·5
% Accumulation control (no DNP)*	74·7	85·7	78·7	56·7	41·6
% Accumulation in presence of DNP*	6·0	15·4	25·9	35·3	38·6
% DNP/% Control	8	18	33	62	93

Organisms grown in medium at pH 7·5

Initial pH value of suspension medium (T_0)	6·0	6·5	7·0	7·5	7·8
Terminal pH value of control (T_2)	6·4	6·6	7·0	7·5	7·6
Terminal pH value in presence of DNP	5·7	6·75	7·0	7·35	7·65
% Accumulation control (no DNP)*	33·2	60·0	35·9	29·3	21·7
% Accumulation in presence of DNP*	0·0	11·3	23·8	20·4	18·4
% DNP/% Control	0	19	66	70	85

* In all cases the standard deviations were less than 2%.

DISCUSSION

The data presented indicate that the accumulation of orthophosphate varied with the pH value of the suspension medium, with a maximum at pH 6·5. Van den Honert (1933) found that phosphate accumulation in sugar cane varied with the pH value of the medium in a manner suggesting that the rate of accumulation was dependent on the concentration of the monobasic phosphate ion. He offered an explanation based on the assumption that the dibasic ions could not be absorbed. In support of such a hypothesis, van den Honert found that accumulation at high phosphate concentrations was independent of changes in the acidity of the medium. The data concerning phosphate accumulation by *Tetrahymena pyriformis* cannot be explained solely on the basis of the ionization state of the phosphate ion. Such an explanation would not account for the sharp maximum in accumulation at pH 6·5. Even though monobasic phosphate ions are in greater concentration at pH 6·0, accumulation is lower. The pH optimum could be explained if the mechanism concerned with orthophosphate entry involves specific sites in the cellular membrane which are sensitive to hydrogen ions. Thus, it is possible that two pH-dependent factors might be involved, the state of the phosphate ion and that of a carrier, the combined effect of these producing an optimum at pH 6·5. Goodman & Rothstein (1957) studied the effect of extracellular pH value on radiophosphate accumulation in yeast and reported that accumulation was maximal when the pH value of the suspension medium was 6·5.

The amount of radiophosphate accumulated by Tetrahymena cannot be accounted for on the basis of free diffusion or catalysed permeability. The total volume of organisms used in these experiments was about 0·030 ml., while the volume of the suspension medium was 20 ml. At the end of the 2 hr. incubation period about 30% of the radiophosphate remained in the medium; thus phosphate had been concentrated by the organisms by a factor greater than 900. It was shown by Slater (1957) and confirmed in this laboratory (Conner, 1958) that no detectable efflux of radiophosphate occurred from Tetrahymena under the conditions used. This removal of radioactivity from the medium probably represents true accumulation and not phosphate ion exchange. This is an accumulation against a concentration gradient and can be explained on the basis of an active transport mechanism.

In Tetrahymena the most pronounced influence of a given concentration of DNP in inhibiting growth or in lowering radiophosphate accumulation occurred in the acid range. Since DNP is a weak acid and is less dissociated in an acidic medium, it seems possible to correlate the degree to which DNP lowers phosphate accumulation with the concentration of free acid. The experiments of DeDeken (1955) and Cross, Taggart, Covo & Green (1949) indicate that the ability of substituted phenols to influence biological processes is a function of the phenolic configuration, the mobility of the phenolic proton, and an inverse function of pK.

Hamburger & Zeuthen (1957) reported DNP to have a greater influence on respiration of *Tetrahymena pyriformis* in an acidic medium and suggested that the free acid is the physiologically active form. It seems, however, that an equally plausible explanation might be to assume that undissociated DNP has the greater ability to penetrate. If only the free acid were active or if both forms penetrated the cellular membrane to an equal extent, it would be necessary to postulate that intracellular pH varies with the pH value of the suspension medium in order to explain the observed results on respiration, growth and phosphate accumulation. Caldwell (1957) found that the extent to which DNP affected metabolic activity in the giant axons of *Loligo forbesi* depended on the concentration of the substituted phenol in the axoplasm, which in turn was dependent on the pH value of the solution bathing the axon. A similar situation might exist with Tetrahymena.

The pH optimum for phosphate accumulation was not seen when DNP was added. Thus in the presence of DNP some other factor becomes rate limiting. A lowered utilization or an intracellular increase of orthoposphate could account for the lowered phosphate accumulation and for the disappearance of the optimum in accumulation at pH 6·5. Such an explanation would be compatible with the current concept of the action of DNP as an uncoupling agent of oxidative phosphorylation and as a stimulant of ATPase activity (Lardy & Wellman, 1953). However, there are other reasonable explanations that could account for the results obtained. It is possible that DNP interferes with an active transport mechanism involved in phosphate entry: directly by combining with a carrier or indirectly by decreasing the supply of energy required for active transport (Conway, 1955). Work is in progress to test the validity of the above hypotheses.

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A Disease of Finnock Due to *Vibrio anguillarum*

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(*Received* 10 *August* 1960)

SUMMARY

A disease of finnock (immature *Salmo trutta*) is described. The causal organism was found to be a Gram-negative, motile, curved rod which was also pathogenic to eels, perch, plaice and saithe. On comparison with other pathogenic vibrios from fish it was apparent that the organism was a variant of *Vibrio anguillarum*; the name *V. anguillarum* type C is suggested for it.

INTRODUCTION

Vibrio anguillarum has long been known to be responsible for red disease of eels, but the descriptions of the organism are various and incomplete, and it has been omitted from *Bergey's Manual of Determinative Bacteriology*, 7th ed. Nybelin (1935) collected and classified all the strains available at that time, and it is his description which will be used here for comparative purposes.

A disease of young Pacific salmon held in sea-water ponds was reported by Rucker, Earp & Ordal (1954) as due to a marine species of Vibrio. The fish develop extensive haemorrhages in the muscle and internal organs, and the disease which begins in April or May when the temperature reaches about 50° F. continues throughout the summer. Hoshina (1956) described a disease of rainbow trout which occurred from late autumn till spring in Japan. It affected both young fish and adults, and was characterized by the production of boil-like lesions or haemorrhages in the muscle. In 1957 Hoshina gave a fuller description of the causal organism which he named *V. piscium* var. *japonicus*. Rucker (1959) cited various outbreaks of disease due to *Vibrio* spp. in Pacific salmon, rainbow trout and steel head trout. The organism was found to be different from *V. comma* and was not pathogenic to warm-blooded animals.

Smith (1959) reported an outbreak of disease in finnock in Scotland and stated that it was due to a *Vibrio* species. The purpose of this paper is to describe the organism isolated from these finnock and by comparison with other similar organisms to show that it is *V. anguillarum*.

SPECIMENS EXAMINED

During the 6-year period (1954–59) 15 finnock and one sea trout were examined (Table 1).

The dead fish were usually found in late summer or early autumn and always in the tidal part of the river. It was also evident that no one sign of disease was common to all the specimens, but the same organism was isolated in every case. Very often this organism grew in pure culture from the spleen, liver, kidney, heart blood and, sometimes, muscle. Sections were also examined but no consistent pathological change was revealed.

The same organism was isolated from a salmon found dead in the River Foyle in August 1959 (Edwards, O'Kelly, Napier & Fletcher, 1960) and the disease was confirmed in finnock and sea trout of the River Ythan as well as in finnock from the River Dee in the summer of 1960.

Table 1. *Specimens examined*

Date	Source	No. of specimens seen	No. of specimens examined	External appearance
September 1954	River Dee, George VI Bridge	3–4	1	Right pectoral fin congested; vent haemorrhagic
September 1955	Cowie Burn	1*	1	Vent distended; small abrasion on left operculum
August 1957	River Dee, Pots and Fords Fishings	*c.* 30	1	Vent haemorrhagic; haemorrhagic area on left flank
August 1958	River Dee, Victoria Bridge	*c.* 20	11	Varied from no sign of disease to congestion of fins, haemorrhage of vent and haemorrhagic areas on flanks
July 1959	River Dee, Torry Beach	2	2	Haemorrhagic swelling on right flank of one and right shoulder of the other

* Sea trout.

DESCRIPTION OF THE ORGANISM

(All tests carried out at 22° unless otherwise stated.)

Morphology. The organism is a small Gram-negative rod ($2–2·5\mu \times 1\mu$) with a tendency to be curved, especially when direct smears or sections from fish are examined. It is motile by a polar flagellum, non-sporing, non-acid-fast and non-capsulate. Cultures on sea-water agar for 6 days or more show a variable number of oval bodies. These when examined in the phase-contrast microscope appear to have polar granulations. They are similar to the bodies mentioned by Liston (1955) in his description of gut group vibrios.

Growth on nutrient agar plate. Colonies are round, raised, mucoid, translucent and greyish yellow in colour. This organism is non-luminous.

Growth on sea-water agar plate. Colonies are round, raised, translucent but whiter than on nutrient agar. They are also of a more watery consistency.

Growth on nutrient agar slope. Raised mucoid, translucent, greyish yellow, tending to coalesce if growth is heavy.

Growth on nutrient agar stab. On the surface there was a spreading shiny mucoid growth and down the line of the stab there was a sharply defined area of growth. This showed the organism was a facultative anaerobe.

Growth on blood agar. The organism does not produce haemolysis on horse blood agar.

Growth in nutrient broth. A turbidity develops overnight and this is followed by the production of a pellicle and formation of a deposit. This deposit becomes viscid later.

Growth on Loeffler's serum slope. The organism grows as a smooth, yellow, shiny layer. The serum is liquefied.

Growth on potato slope. On alkaline potato slope the growth is smooth, shiny and yellow.

Biochemical reactions. The organism produces acid but no gas from glucose, fructose, sucrose, maltose, trehalose, starch, dextrin, glycogen, mannitol, sorbitol; slight acid but no gas from galactose and no acid or gas from arabinose, rhamnose, xylose, lactose, cellobiose, raffinose, inulin, glycerol, dulcitol, inositol, amygdalin and salicin. When tested by Hugh & Leifson's (1953) method the organism was found to belong to Group III*a*, i.e. anaerogenic fermenter. It is methyl red negative, produces acetylmethylcarbinol; does not produce indole; does not grow on Koser's citrate medium; does not produce hydrogen sulphide on ZoBell's medium; produces catalase on nutrient agar; reduces nitrate to nitrite; liquefies gelatin but has no action on filter-paper. In litmus milk a clot is produced and this is followed by proteolysis and reduction.

Table 2. *Pathogenicity experiments*

Fish	No.	Route of injection	Time to die (days)	External symptoms
Finnock	3	intraperitoneal	1–6	Two showed hamorrhage of the vent; one showed no external symptoms
Finnock	1	intramuscular	2	Inflammation of muscle at site of injection
Eels	4	intramuscular	2–7	Haemorrhagic swelling at site of injection and congestion of muscle
Brown trout	2	intraperitoneal	4–6	None
Perch	2	intraperitoneal	2	None
Plaice	1	intramuscular	22	Swollen haemorrhagic area about the size of a penny with a bluish black centre. Haemorrhage in peripheral muscle
Saithe	2	intraperitoneal	2–6	One showed no symptoms but the other developed a greyish area on its dorsal surface.

In all cases the organism was re-isolated after death but in no case was it found in an inoculated or uninoculated control.

Reaction to antibiotics. Evans's 'Senstests' were used. The organism was resistant to 2·5 i.u. penicillin but sensitive to 10 μg. terramycin. It was also sensitive to the vibriostatic agent 0–129 (2-4-diamino-6-7-di-isopropyl pteridine).

Metabolic properties. When the tap water used to make up peptone water was replaced completely or partially by sea water, the resulting growth was increased and appeared optimal when the concentration of salt in the medium was approximately 2%. The organism grew slowly at 5° and at 34° but not at 37°. On salt containing media it grew better at 22° than at 15°, but on ordinary media the final growth was more or less equal at 15° and 22°. It grows from pH 6·0 to 9·0 with an optimum at 6·8. All these results are based on nephelometer readings. The organism will withstand heating to 40° for 10 min. but not to 45° for the same time.

Pathogenicity. Table 2 shows that the organism kills five species of fish including freshwater anadromous, catadromous and marine species.

DISCUSSION

When this organism is classified according to Skerman (1957) it is found to belong to the family *Vibrio* if it is treated as a curved rod or to the *Pseudomonas* family if a straight one. Haynes & Burkeholder (1957) state that the distinction of the straight rods of the *Pseudomonas* from the curved rods found in *Vibrio* is difficult as the curvature of the organism sometimes depends on the medium employed. It has, however, been found that the pseudomonads are oxidative in their action on carbohydrates while the vibrios are fermentative (Hugh & Leifson, 1953). This organism is fermentative, i.e. it can produce acid from carbohydrates under anaerobic conditions so that on this criterion it should belong to the *Vibrio* genus. In addition, it is sensitive to the 0–129 which Shewan, Hodgkiss & Liston (1954) state is a vibriostatic agent. This is further evidence that the organism is a *Vibrio* species.

For comparison the following cultures which had been isolated from diseased fish were obtained and examined.

(1) *Vibrio anguillarum*, National Collection of Marine Bacteria No. 6 (Torry Research Station, Aberdeen), isolated by Bagge & Bagge (1956) from diseased codling in Denmark.

(2) *V. piscium* var. *japonicus* isolated by Hoshina (1956) from rainbow trout in Japan.

(3) *V. ichthyodermis* isolated by Hodgkiss & Shewan (1950) from a plaice. This was initially called *Pseudomonas ichthyodermis* but the authors now consider it to be a *Vibrio*.

Vibrio anguillarum when injected intramuscularly into eels caused death in 2–6 days. It produced haemorrhage at the site of inoculation in two, swelling and congestion in the third and no symptoms in the fourth. The organism was re-isolated however from them all, and in every case was found in sections of the spleen, liver or muscle. Intramuscular injection of perch gave rise to swellings and the perch died in 6–10 days. The organism was re-isolated from one and found in liver and muscle sections. Trout also succumbed to intraperitoneal injection in 16–21 days. The peritoneum of one was distended and contained much reddish brown fluid while the vent of the second was slightly distended and haemorrhagic. The organism was re-isolated after death and observed in sections of muscle of both trout.

Neither *Vibrio piscium* var. *japonicus* nor *V. ichthyodermis* was pathogenic for eels but both killed one of four trout inoculated intraperitoneally. In both these cases the organism was re-isolated. This low pathogenicity may well be due to the fact that these organisms have been *in vitro* culture for some considerable time.

The above three cultures were also studied morphologically and biochemically, and were found to be very similar to the organism isolated from the finnock.

Vibrio anguillarum differed from the finnock culture only in its ability to ferment arabinose, glycerol and cellobiose and its inability to ferment starch. It produced indole and grew on Koser's citrate medium. *V. piscium* var. *japonicus* differed in its action on arabinose, starch inositol and cellobiose and *V. ichthyodermis* by its action on sorbitol, glycerol, starch and cellobiose, its inability to produce acetylmethylcarbinol and its ability to produce indole.

The descriptions of *Vibrio anguillarum* in the literature vary to some extent, but Nybelin (1935) described *V. anguillarum* as a Gram-negative curved bacillus which was actively motile, non-sporing and non-capsulate. It liquefied gelatin in a similar manner to the cholera bacillus, produced acid but no gas from glucose or maltose, did not produce hydrogen sulphide, had no reducing properties, was haemolytic (only a few strains examined); and was a halophile. He subdivided this species into two subtypes A and B. A was able to produce acid but no gas from sucrose and mannitol and to produce indole, whereas B had no action on these sugars and did not produce indole. Bagge & Bagge (1956) describe their isolate as *V. anguillarum* type A on this basis.

Hoshina (1957) ascribed the name *Vibrio piscium* var. *japonicus* to the organism he isolated from rainbow trout as he thought it resembled the organism *V. piscium* which David (1927) had isolated from carp. It seems, however, that it is more likely to be a variant of *V. anguillarum* as its growth characteristics are almost identical with those of *V. anguillarum*, and it does not produce indole or hydrogen sulphide to any great extent (I could find neither), whereas *V. piscium* produces them both; lastly Hoshina's culture and *V. anguillarum* both produce acid but no gas from sugars and reduce nitrates to nitrites, while *V. piscium* can neither ferment carbohydrates nor reduce nitrates. It would therefore seem that this organism would be more appropriately named *V. anguillarum*.

Likewise the organism described here falls into Nybelin's general classification except for the haemolysis of rabbit blood which was not studied and the reducing properties which this organism has. It appears, however, that this organism is intermediate between A and B. Nybelin (1935) did study such a strain. He obtained it from Schäperclaus who isolated it at Lübeck in 1932. This strain was able to produce acid from both sucrose and mannitol but could not produce indole. Nybelin considered it unnecessary to postulate a third type to cover this strain, as it might have been a weak indole producer, but the strain from finnock and that of Hoshina seem to be identical with the Lübeck strain and so should be called *V. anguillarum* type C.

No attempt has been made to study the antigenic structure of the strains considered here, as previous workers have shown that the structure is complex, and Nybelin (1935) found that cross-reactions rarely occurred in organisms isolated from different epizootics.

Strains of this organism have been lodged with the National Collection of Marine Bacteria at Torry Research Station, Aberdeen.

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The Sugar Composition of Streptococcal Cell Walls and its Relation to Haemagglutination Pattern

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SUMMARY

The sugar composition of cell walls of various streptococci and pneumococci was investigated by paper chromatography. Rhamnose was found in the walls of most streptococci bearing Lancefield group haptens but was not identified in the walls of pneumococci or most 'viridans' streptococci. In the group as a whole galactose and glucose were more regularly present than rhamnose. A significant correlation was found between the distribution of rhamnose and haemagglutination pattern, as determined by the presence of a widely distributed red cell sensitizing antigen and the production of an agent capable, like the receptor-destroying enzyme of the influenza virus, of modifying the antigenic properties of the red-cell surface.

INTRODUCTION

In recent years considerable advances have been made in our knowledge of the composition and structure of the bacterial cell wall. These advances were made possible by the development by Salton and others (Salton & Horne, 1951; Salton, 1952; Cummins & Harris, 1956) of satisfactory techniques for the preparation of purified cell-wall homogenates. On *a priori* grounds it seems likely that cell-wall composition would have considerable taxonomic value. This possibility is borne out particularly by the finding that the cell walls of Gram-negative bacteria show, as far as they have been examined, a wider range of amino acids and a higher lipid content than do those of Gram-positive organisms (Salton, 1952, 1953) and of generic differences amongst various Gram-positive bacteria in cell-wall sugar composition (Cummins & Harris, 1956, 1958).

An analysis of the cell-wall composition of various streptococci was reported by Cummins & Harris (1956). These workers found rhamnose to be a constituent of the cell walls of strains of streptococci of Lancefield groups A to G; from these results it seemed possible that rhamnose, which they found to be absent from the cell walls of staphylococci and certain other species, might be characteristic of the streptococci as a whole. They noted, however, differences between different strains of streptococci in their content of other sugars, and were in general of the opinion that the sugar and amino sugar composition of the cell wall was of taxonomic significance at species level. A quite different approach to the taxonomy of the streptococci was described by Stewart, Steele & Martin (1959). This proposed that these organisms might be classified by the haemagglutination technique on the basis of the distribution of various red cell sensitizing antigens and of an agent—cell-modifying agent (CMA)—possessing properties similar to those of the receptor-destroying enzyme of the influenza virus.

The present communication reports the results of an investigation by paper chromatography into the sugar composition of the cell walls of various streptococci and pneumococci and considers in particular the relationship between cell-wall sugar content and haemagglutination pattern.

METHODS

Organisms. At least one member of each Lancefield group A to Q examined was from the National Collection of Type Cultures (N.C.T.C., Colindale, London, N.W. 9). Two of the group K strains produced mucoid colonies on 5 % (w/v) sucrose agar and may therefore be classified as *Streptococcus salivarius*. The strains of groups R and S examined were made available to us by the courtesy of Dr C. E. de Moor (Utrecht). The majority of the 'viridans' and group K strains were laboratory isolates from upper respiratory sources (throat swabs, sputa, saliva). They were selected in the first instance on the basis of the production of a viridans change on human blood agar incubated aerobically and were insensitive to 1 in 5000 ethyl hydrocuprein, were deoxycholate insoluble and did not produce mucoid colonies on sucrose agar. All strains classified as 'viridans' streptococci in addition gave negative tube precipitin reactions with antisera for Lancefield groups A to N. The Lancefield groups were determined on 48 hr. cultures in 0·5 % (w/v) glucose digest broth extracted at 100° with 0·05 N-HCl. The precipitating sera used were those issued commercially by Burroughs Wellcome and Co.

Group O serum was also used routinely in our tests, but doubts about its specificity were raised by the finding of what appeared to be an excessively high proportion of reactions with 'viridans' strains. On further investigation the serum was found to react with a number of NCTC strains belonging to various other Lancefield groups. On examination by the Ouchterlony plate diffusion technique against the NCTC group O strain—8029—the serum was found to give a number of distinct precipitation lines. Pending fuller investigations the designation group O has therefore been withheld from all strains except NCTC 8029.

The pneumococci examined included one strain of each of types 2 (NCTC 7466), 14 and 16 and two strains of type 3. The remaining two strains were not typed.

Cell-wall analysis. Cultures for cell-wall analysis were obtained by overnight growth at 37° in Hartley's digest broth containing 0·2 % (w/v) $NaHCO_3$ and 0·5 % (w/v) glucose. Five hundred ml. of culture were usually sufficient for examination except in the case of the pneumococci when twice this volume was found necessary to give a satisfactory bacterial mass. The organisms were harvested by centrifugation at 3000 *g* and washed twice in 0·9 % (w/v) saline.

The method used for the preparation of cell walls was essentially that of Cummins & Harris (1956), involving disintegration of the organisms by shaking in a Mickle disintegrator with no. 12 ballotini glass beads. Disintegration was considered complete when no intact organisms could be seen in Gram-stained films. The glass beads were removed by passage through a coarse sintered glass filter. The cell walls were separated by differential centrifugation and purified by washing with distilled water, followed by treatment with trypsin and ribonuclease. The cell walls were then sedimented by centrifugation, washed in water and resuspended in water. Peptic digestion was omitted since control experiments with a number of strains showed no difference between pepsin-treated cell walls and walls which had not been treated with pepsin.

Random samples were examined from time to time in a Beckmann spectrophotometer to detect the presence of contaminating nucleotide material from the cytoplasm (Salton & Horne, 1951; Barkulis & Jones, 1957). In no case was any peak observed in the 260 mμ region.

Conditions and treatment. The method of hydrolysis employed followed that used by Cummins & Harris (1956) viz. treatment with 2N-H_2SO_4 at 100° for 2 hr. in sealed tubes. The hydrolysate was neutralized with $Ba(OH)_2$ and then dried over P_2O_5 in an Edwards centrifugal freeze-drying apparatus. Approximately 30 mg. dry weight of cell wall was used in each test; where this amount was not available the final volume of the hydrolysate was adjusted accordingly—a constant volume of this being applied to each chromatogram.

One-dimensional descending chromatograms on Whatman no. 4 paper with the solvent ethyl acetate + pyridine + water of Jermyn & Isherwood (1949) were found to resolve all the sugars present provided that the solvent front was allowed to run well off the paper. The sugars were revealed with aniline hydrogen phthalate, and hexosamines with the Elson and Morgan reagent. No attempt was made to separate individual hexosamines.

Haemagglutination techniques. Cultures for haemagglutination tests were obtained by growth for *c.* 18 hr. in Hartley's digest broth. The cultures were centrifuged until visibly clear and the supernatant fluids thus obtained used for cell treatment. For the preparation of absorbing suspensions 48 hr. cultures in digest broth containing 0·5 % (w/v) glucose were used. Human group O red cells, washed three times in saline before use and resuspended in saline to give a *c.* 5 % suspension were used for cell treatment. Treatment was carried out by incubating mixtures of equal volumes of cell suspension and of supernatant fluid for 2 hr. at 37°. At the end of this time the cells were sedimented by centrifugation, washed in saline and resuspended to give a 5 % suspension. For the haemagglutination tests proper one drop of cell suspension and one drop of serum dilution were mixed and the mixtures allowed to stand for 30 min. at room temperature; they were then examined for agglutination by inspection of the deposit with a × 10 microscope ocular.

The serum used for the detection of the Hickey antigen was obtained by immunization of a rabbit with the deposit from a culture of *Streptococcus pyogenes* in 0·5 % (w/v) glucose digest broth. The serum was heated to 56° for 30 min. and, in order to remove naturally occurring T agglutinins, was absorbed with red cells treated with the supernatant fluid of a digest broth culture of a 'viridans' streptococcus which produced the cell-modifying (receptor-destroying) enzyme. For test purposes the serum was used in a dilution of 1/20.

The serum used for the detection of cell-modifying activity was obtained by immunization of a rabbit with cells treated with allantoic fluid from chick embryos infected with the FMI strain (type AI) of influenza virus. The serum was heated to 56° for 30 min. and was heavily absorbed with normal red cells to remove species agglutinins. For test purposes it was used in a dilution of 1/40.

RESULTS

Cell-wall composition

The results of the cell-wall analyses are shown in Table 1. It will be seen that though in agreement with the findings of Cummins & Harris (1956) rhamnose was a component of the cell walls of the streptococci of groups A to G examined; its presence was not a general property of the streptococci since it was absent from the group O strain (NCTC 8029), from a number of group K strains, from the majority of the ‘viridans’ streptococci and from all the pneumococci. The two strains of *Streptococcus salivarius* classified as group K were rhamnose positive. The absence of rhamnose from the cell walls of pneumococci is consistent with the findings of Smith, Mills, Harper & Galloway (1957) who failed to detect this sugar in the cellular polysaccharide obtained by enzymic digestion of whole pneumococci.

Table 1. *Cell-wall sugar composition of various streptococci*

	Sugars			
	Gal + Gl +	Gal − Gl +	Gal + Gl −	Gal − Gl −
	Strains			
Rhamnose-positive	A (1), D (2), F (3), H (2), K (10), L (1), M (1), P (1), Q (1), R (2), S (2), ‘Viridans’ (11)	E (1), H (1), N (1), ‘Viridans’ (1)	B (1), D (1), G (1)	A (4), C (1)
Rhamnose-negative	K (3), O (1), ‘Viridans’ (21), Pneumococci (7)	K (1)	‘Viridans’ (3)	

Gal = galactose; Gl = glucose. Hexosamine present in all strains. Figures in parentheses indicate number of strains examined.

Surprisingly, considerably less variation was found in the group as a whole in the distribution of galactose and glucose than in that of rhamnose. With the exception of four strains of *Streptococcus pyogenes* and one group C strain, all strains possessed one or other of these sugars and most possessed both. From a taxonomic point of view it is significant, however, that of the seven groups of which more than one strain was examined, intra-group variation in either galactose or glucose content was encountered in four groups, namely A, D, H and K. The discovery of a group A strain which possessed both galactose and glucose is of interest in view of the fact that previous strains of this species which have been reported were found to be deficient in both these sugars (Barkulis & Jones, 1957; Cummins & Harris, 1956; McCarty, 1952; Salton, 1953; Schmidt, 1952). Both galactose and glucose were regularly present in the pneumococci and appeared to be somewhat more consistently present, though not significantly so, in the ‘viridans’ streptococci than in the streptococci identified as possessing Lancefield group haptens. Unlike Cummins & Harris (1956), who detected mannose in the cell walls of one group E and two group D strains, we have failed to identify mannose in any of our strains.

Relationship of cell-wall sugar content to haemagglutination pattern

The relationship between cell-wall sugar content and haemagglutination pattern as determined by the reactions with the CMA and Hickey sera is shown in Table 4. Tests for the Hickey antigen were carried out in the first place with the supernatant fluids of digest broth cultures. Strains which gave negative results in these tests were then retested by an absorption technique. For this purpose the sediments from 48 hr. cultures in digest broth containing 0·5 % (w/v) glucose were used, the sediment from 20 ml. culture being used to absorb 1 ml. unabsorbed Hickey serum diluted 1/20. Absorption was for 30 min. at room temperature. In each case two consecutive absorptions of the serum were carried out, the same amount of culture being used for the second absorption. The unabsorbed serum and samples of serum after each absorption were then titrated against red cells treated with supernatant fluid of the Hickey (*Streptococcus pyogenes*) strain. The results obtained with this method were quite clear cut, most Hickey-positive strains removing the antibody completely after the first absorption, and Hickey-negative strains causing at most a twofold decrease in titre after two absorptions. A typical absorption result is shown in Table 2.

Table 2. *Absorption of Hickey serum with representative strains of streptococci*

Figures given are reciprocals of serum dilution causing agglutination.

	Test cocci		
	Treated with Hickey supernatant fluid		Untreated
	Reciprocals of serum dilution		
	1st absorption	2nd absorption	
BI (CMA-positive viridans)	640	320	< 40
MM (CMA-positive group K)	320	320	< 40
NCTC 8029 CMA-positive group O	80	< 40	< 40
44 CMA-negative group K	< 40	.	< 40
Unabsorbed	640		< 40

CMA = cell-modifying agent.

In the earlier work, in which the distribution of the Hickey antigen was studied only in the supernatant fluids of digest broth cultures (Stewart *et al.* 1959), a very high negative correlation was found between the distribution of the antigen and that of the cell-modifying enzyme. With the more rigorous technique used in the present work it was found, however, that strains which give negative results for the Hickey antigen in digest broth might yet be shown by the absorption technique to possess it. One such culture was the group O strain (NCTC 8029). On repeated testing of this strain, however, occasional positive haemagglutination reactions were obtained with the supernatant fluids of glucose digest broth cultures. (For tests with glucose digest broth the red cells were suspended in phosphate buffered saline at pH 7).

The distribution of the four haemagglutination patterns determined as described in the preceding paragraph amongst the various streptococci examined is shown in Table 3. It will be seen that, excluding certain group K strains the Hickey antigen was present in all the streptococci bearing Lancefield group haptens examined, and that all except two of these were CMA negative. This distribution is in sharp contrast to that found in the pneumococci, all of which were Hickey-negative CMA-positive. In contrast, the 'viridans' streptococci showed a much greater diversity of haemagglutination type.

Table 3. *Haemagglutination patterns of strains examined*

	Haemagglutination type			
	Hickey + CMA −	Hickey − CMA +	Hickey + CMA +	Hickey − CMA −
	Number of strains			
Streptococci of groups A to S (excluding K)	26	0	2	0
Group K streptococci	9	5	0	0
'Viridans' streptococci	12	14	6	4
Pneumococci	0	7	0	0

CMA = cell-modifying agent.

From the results shown in Table 4 it will be seen that there was a considerable correlation, amongst the streptococci, between the presence of rhamnose as a cell-wall component and the haemagglutination type. This is best seen in the relative frequencies of the Hickey-positive CMA-negative and Hickey-negative CMA-positive types in rhamnose-positive and rhamnose-negative strains. Thus of 49 rhamnose-positive strains 41 were Hickey-positive CMA-negative and 6 were Hickey-negative CMA-positive. The rhamnose-negative strains, on the other hand, showed a quite different haemagglutination pattern; of 29 of such strains examined 6 were Hickey-positive CMA-negative and 13 were Hickey-negative CMA-positive ($\chi^2 = 20{\cdot}4$. $P < 0{\cdot}001$). This correlation was even greater when the results with digest broth cultures alone were considered. In this case 40

Table 4. *Relationship between streptococcal cell-wall sugar content and haemagglutination (HA) type*

HA type		Cell-wall sugars					
		Rhamnose		Galactose		Glucose	
		+	−	+	−	+	−
Hickey	CMA	Number of strains					
+	−	41	6	38	9	37	10
−	+	6	13	18	1	19	0
+	+	2	6	8	0	7	1
−	−	0	4	4	0	4	0

CMA = cell-modifying agent.
Results obtained with pneumococci excluded from this table.

of the rhamnose-positive strains were Hickey-positive CMA-negative and 6 were Hickey-negative CMA-positive, while of the rhamnose-negative strains 2 were Hickey-positive CMA-negative and 17 were Hickey-negative CMA-positive.

Of the 14 group K strains examined 9 were rhamnose-positive Hickey-positive CMA-negative, 4 were rhamnose-negative Hickey-negative CMA-positive, and one was rhamnose-positive Hickey-negative CMA-positive.

DISCUSSION

Our results have shown that rhamnose, though present in the cell walls of most streptococci possessing Lancefield group haptens, is not characteristic of the streptococci as a whole. In particular, it was found to be absent from the walls of the definitive group O strain examined, from those of certain group K streptococci and from those of the majority of viridans streptococci. The question therefore arises as to whether, amongst the streptococci, the presence of rhamnose as a cell-wall component is of importance as a criterion of phylogenetic relationship. Its presence in most streptococci bearing Lancefield group haptens and its absence from the pneumococci would suggest that it is. This view is reinforced by the finding that the distribution of rhamnose has a significant relation to haemagglutination pattern as determined by the presence of the Hickey antigen and the production of the cell-modifying enzyme. One is in fact tempted to see in this group of organisms two basic types from which others might have arisen by a process of hybridization namely: (1) a rhamnose-positive Hickey-positive CMA-negative type typical of the majority of the Lancefield group streptococci; (2) a rhamnose-negative Hickey-negative CMA-positive type characteristic of the pneumococci. These two types have in fact accounted for just over two-thirds of the strains of streptococci examined.

The status of group K strains as defined by these reactions is of interest. Group K streptococci have already been shown to be heterogeneous in respect of their capacity to produce mucoid colonies on sucrose agar—a property which is accepted as the definitive criterion of the species *Streptococcus salivarius* (Williams, 1956). In fact from the work of Williams the production of mucoid colonies on sucrose agar appears to be better correlated with various other physiological properties than is the possession of the K hapten. Group K strains are also heterogeneous in their haemagglutination patterns. The introduction of rhamnose as an additional parameter introduces still further heterogeneity into the group.

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Morphological and Biochemical Features of 'Atypical' Mycobacteria

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SUMMARY

Morphological and biochemical features of 42 strains of 'atypical' mycobacteria and one strain of *Mycobacterium tuberculosis* were studied. Of the 42 atypical mycobacteria, 16 were originally classified in Runyon's group I, 4 in group II, 19 in group III, and 3 in group IV. The characteristics studied were bacillary morphology and staining properties on Kirschner and Löwenstein–Jensen media; colonial morphology on 7H-10 agar medium; pigmentation in the dark and after exposure to light; rate of growth and temperature requirements, with different methods of inoculation; growth on blood and nutrient agar plates, and in gelatin stabs; catalase activity on drug-free and on isoniazid-containing media; nicotinic acid (niacin) production. The sensitivity of the majority of the strains to 6 chemotherapeutic drugs was tested. The niacin test proved to be the most useful method for distinguishing the atypical mycobacteria from *M. tuberculosis*. In identifying strains of group I, their ability to produce yellow pigment after exposure to light was of most value, and their colonial morphology and their periodic acid-Schiff staining were also helpful. Strains of group II were identified by their ability to form yellow pigment in the dark, by their periodic acid-Schiff staining and by their colonial morphology. Strains of group III were identified by their rate of growth and buff pigmentation. Exceptionally a yellow pigment was formed, and such strains were identified principally by their colonial morphology and periodic acid-Schiff staining. Among the 19 strains classified originally as group III, 3 were reclassified into group IV. Strains of group IV were identified by their ability to grow on blood and nutrient agar plates within 3 days and in gelatin stab cultures within 2 weeks.

INTRODUCTION

In the last few years several publications have appeared dealing with human diseases caused by 'atypical' mycobacteria (Wood, Buhler & Pollak, 1956; Nassau & Hamilton, 1957; Beck, 1959; Lewis *et al.* 1959; Engbaek, Magnusson & Nielsen 1959). These authors, and others mentioned by Nassau & Hamilton (1957), stated that the diseases caused by atypical mycobacteria and by *Mycobacterium tuberculosis* are closely similar. Thus the correct diagnosis and treatment depends upon the precise identification of the causative agent. The identification of an infection by *M. tuberculosis* in routine diagnostic work often depends on the morphology and the staining characteristics of the bacilli as seen in smears made from clinical specimens and sometimes from cultures, together with the colonial morphology on Löwenstein-Jensen medium. These procedures are liable to considerable misinterpretation. The bacillary morphology and the degree of acid-fastness of *M. tuberculosis* and atypical mycobacteria may be similar. Colonies of *M. tuberculosis* on Löwenstein–Jensen medium may closely resemble those of some other strains of mycobacteria. Several morphological and biochemical tests (reviewed by Freerksen, 1960) have been suggested as additional aids for differentiating atypical mycobacteria from *M. tuberculosis*, such as microcolonial structure (Engbaek, 1952), cord formation (Middlebrook, Dubos & Pierce, 1947; Yegian & Budd, 1953), pigmentation of colonies (Tarshis & Frisch, 1952; Timpe & Runyon, 1954), the neutral red test (Dubos & Middlebrook, 1948; Hauduroy & Posternak, 1949), catalase activity (Middlebrook, 1954) and nicotinic acid (niacin) production (Konno, 1956). No single test has been found to be completely satisfactory for this purpose. Runyon (1959*a*, *b*) proposed a classification of the atypical mycobacteria based mainly on pigmentation of colonies and speed of growth. The purpose of the present investigation was to examine the morphological characteristics and some of the biochemical properties of a series of strains of atypical mycobacteria, classified into Runyon's four groups, and to see whether these characteristics are of value in differentiating these strains from *M. tuberculosis*. All of the strains used were of human origin.

METHODS

Strains

Mycobacterium tuberculosis, 1 strain, H37Rv. Atypical mycobacteria classified according to Runyon (1959*a*): group I, photochromogens (16 strains); group II, scotochromogens (4 strains); group III, Battey type (19 strains); group IV, rapidly growing (3 strains). The origin of the strains is set out in Table 1. All were obtained from human sputum or resected lung. The 13 British strains were obtained from 13 patients who had been examined in various British chest clinics and hospitals. Of these, 4 were described by Selkon & Mitchison (1959). All 29 strains from the United States of America were kindly sent by Dr E. H. Runyon, Veterans Administration Hospital, Salt Lake City, Utah, U.S.A.

Table 1. *The mycobacterial strains examined*

Group	No. of strains tested	Great Britain		United States of America	
		No. of strains	Strain no.	No. of strains	Strain no.
I. Photochromogen	16	10	0523, 0735, 1438, 1526S, 1526R, M1159, M1212, M1246, M1247, A725	6	15, 260, 265, 266, 353, 440
II. Scotochromogen	4	2	0112, M1177	2	50, 251
III. Battey type	19	1	M1208	18	160, 170, 171C, 223, 225, 277, 248, 470, 477, 487, 509, 513, 514, 520, 585, P7, P23, P25
IV. Rapidly growing	3	.	.	3	880, 481, 518

Examination of cultures in Kirschner medium. Kirschner medium (Mackie & McCartney, 1956) was inoculated from Löwenstein–Jensen cultures in screw-capped bottles, which were 3 weeks or less old, and which had not been opened since inoculation. Care was taken not to transfer lumps of bacteria to the Kirschner medium. After incubation for 14 days at 37°, films were made and they were then dried under an ultraviolet lamp for 30 min., placed in methanol for 5 min., and rinsed in water. Ziehl–Neelsen staining was performed in the usual way, with 5 min. decolorization with acid-alcohol (NaCl 20 g., HCl conc. 20 ml., 74° O.P. Spirit 1500 ml., distilled water 400 ml.) and counterstaining with Löffler's methylene blue for 5–15 min. Periodic acid-Schiff staining was performed as described by Csillag (1960).

Examination of cultures on Löwenstein-Jensen medium. Cultures were grown on Löwenstein–Jensen medium for 3 weeks at 37°. Smears, prepared and fixed as described above, were stained by the Ziehl–Neelsen and the Ziehl–Heidenhain (Uyeda, 1955) methods.

Colonial morphology. The morphology of colonies was examined on plates of oleic acid + albumin agar medium (7H-10 medium; Cohn, Middlebrook & Russell, 1959). The plates were inoculated from a 4-week growth on the same medium and incubated for 3–4 weeks at 37° in a hot room where they were occasionally exposed to artificial illumination. The colonies were formolized, and observed with a plate microscope (× 10 magnification).

Pigmentation following incubation in the dark or after exposure to light. Two Löwenstein–Jensen medium slopes were inoculated with each strain. One of these was placed in a sealed box and incubated at 37° in the dark. The other slope was also incubated at 37° and, when growth first appeared, it was exposed to diffuse daylight for 1 hr., re-incubated for 24 hr. and compared for its pigmentation with the slope incubated in the dark. When only minor differences in pigmentation were found between the two slopes, the procedure was repeated on another batch of medium. Those strains which produced yellow or orange pigment when grown in the dark were examined in the same manner, but when growing on 7H-10 medium slopes.

Temperature requirements and rate of growth. The rate of growth at 37° and at 22° was studied on Löwenstein–Jensen medium slopes which had been inoculated with a loop containing a small amount of growth from a 3- to 4-week culture on the same medium ('clumped inoculum' method). With the photochromogens, the rate of growth at the same temperatures was also studied on the same medium which had been inoculated with a 3 mm. loopful of a suspension prepared by shaking about 2 mg. (moist weight) of bacillary mass with 0·4 ml. distilled water in a screw-capped bottle containing glass beads ('dispersed inoculum' method). Cultures were examined at daily intervals (except on Sundays) for 6 weeks.

Growth on blood and nutrient agar. Nutrient agar plates with or without 5 % (v/v) horse blood were inoculated from a 3- to 4-week growth on 7H-10 agar plates. The cultures were incubated at 37° for 3 days.

Growth in gelatin stabs. Nutrient gelatin stab cultures were inoculated with a straight wire from a 3- to 4-week growth on 7H-10 medium plates. The cultures were incubated at 22° for 6 weeks.

The nicotinic acid (niacin) test. Tests for nicotinic acid were carried out as described by Gilani & Selkon (1958), after incubation for 6 weeks.

Catalase activity. Catalase activity (Middlebrook, 1954; Selkon & Mitchison, 1959) was studied on growth on drug-free Löwenstein–Jensen slopes and on slopes containing isoniazid in the sensitivity tests. In studying the catalase activity of isoniazid-resistant variants, Löwenstein–Jensen slopes containing 50 μg. isoniazid/ml. were inoculated with a large amount of bacillary growth, incubated for 4 weeks at 37° and the resulting growth tested.

Sensitivity tests. Sensitivity tests to isoniazid, *p*-aminosalicylic acid, streptomycin and *p*-acetamidobenzaldehyde thiosemicarbazone (thiacetazone) were performed as described by Selkon & Mitchison (1959). The sensitivity to 2-ethylisothionicotinamide (thioamide) and cycloserine was tested in a similar manner, with twofold serial drug dilutions in Löwenstein–Jensen medium. The results of a single test on each strain are reported; unexpected results were not repeated.

RESULTS

Bacillary morphology and staining

The strains were divided into types on the basis of their bacillary morphology ('BM') type and staining properties, as follows.

Group I

BM type Ia (15 strains, all except no. 1526R). Majority of organisms longer than those of *Mycobacterium tuberculosis* H37Rv, and strongly beaded. Tendency to formation of loose cords present, especially in Kirschner medium. Not acid-fast organisms only exceptionally seen with both staining methods, with 5 min. or 15 min. counterstaining. Periodic acid-Schiff staining positive.

BM type Ib (1 strain, no. 1526R). As above, but no tendency to cord formation, either in Kirschner or on Löwenstein–Jensen medium.

Group II

BM type II (4 strains). Size of organisms variable. Majority of organisms beaded. Cord-formation not observed in either medium. Not acid-fast organisms only exceptionally seen with both staining methods. Periodic acid-Schiff staining positive.

Group III

BM type IIIa (10 strains; all nos. except those listed as types IIIb–IIId below). Size of organisms nearly uniform and shorter than those of *Mycobacterium tuberculosis* H37Rv. Beaded or uniform staining. Tendency to cord formation not observed. Not acid-fast organisms rarely seen in growth on Kirschner medium. More not acid-fast organisms seen in growth on Löwenstein–Jensen medium, especially when stained either by the Ziehl-Neelsen method, with 15 min. counterstaining, or by the Ziehl–Heidenhain method. Periodic acid-Schiff staining negative.

BM type IIIb (7 strains, nos. P7, P23, 170, 223, 277, 487, M1208). As for type IIIa, but organisms not uniform in size; filaments, rods and coccoid forms being seen in the same smear. Nearly half of the organisms not acid-fast in growth on Löwenstein–Jensen medium, with all staining methods. Majority of organisms not acid-fast in Kirschner medium. Periodic acid-Schiff staining negative.

BM type III*c* (1 strain: no 171). As for type IIIb, but, after 8 preliminary serial subcultures at monthly intervals on Löwenstein–Jensen medium, exclusively not acid-fast organisms seen in both media. Organisms of types IIIa, IIIb and IIId retained acid-fastness during similar passages. Periodic acid-Schiff staining negative.

BM type IIId (1 strain: no. 160). As for type IIIa, but tight cords present in growth from Kirschner medium. Periodic acid-Schiff staining negative.

Group IV

BM type IV (3 strains). Size of organisms similar to those of *Mycobacterium tuberculosis* H37Rv. Staining uniform. Tight cords present. Not acid-fast organisms only exceptionally seen in both media, with both staining methods. Periodic acid-Schiff staining negative.

Mycobacterium tuberculosis (H37Rv). Size of organisms typical for *M. tuberculosis*. Staining uniform or beaded. Tight cords present. Not acid-fast elements only exceptionally seen in both media, with both staining methods. Periodic acid-Schiff staining negative.

Colonial morphology

The strains were divided into types on the basis of their colonial morphology ('CM' types) on 7H-10 medium, as follows.

Group I

CM type Ia (13 strains, all nos., except those listed as type Ib). Circular, differences in size slight, low convex, finely granular surface, slightly crenated edge, central area of opacity, pigmented buff, butyrous.

CM type Ib (3 strains, nos. 440, 0735, 1526R). Circular, differences in size slight, raised, coarsely granular surface, crenated edge, some colonies umbonate, heaping between adjacent colonies sometimes seen, pigmented buff, friable, difficult to emulsify.

Group II

CM type II (4 strains). Circular, nearly uniform in size, high, convex, smooth surface, uniform structure, entire edge, pigmented yellow, butyrous.

Group III

CM type IIIa (11 strains: all nos. except those listed as types IIIb–IIIe below). Circular, size variable, domed, smooth surface, uniform structure, entire or slightly crenated edge, pigmented buff, butyrous.

CM type IIIb (2 strains, nos, 514, 520). As for type IIIa, but pigmented yellow.

CM type IIIc (1 strain, no. 513). As for type IIIa, but colonies irregular in shape.

CM type IIId (1 strain; no. 470). As for type IIIa, but size uniform and central area of opacity.

CM type IIIe (4 strains: nos. 160, 170, 225, 509). Circular, size very variable, effuse, umbonate, finely granular surface, entire or crenated edge, pigmented buff, friable.

Group IV

CM type IV (3 strains). Circular, size variable, low convex, coarsely granular surface, crenated edge, some colonies in each strain with centre umbonate, heaping between adjacent colonies seen, pigmented buff, friable.

Mycobacterium tuberculosis. Circular, size slightly variable, low convex, coarsely granular surface, crenated edge, centre umbonate, heaping between adjacent colonies seen, pigmented buff, friable.

Pigmentation

Pigmentation was observed on colonies grown systematically in the dark or exposed to light, with the following results.

Group I. All strains buff, when grown on Löwenstein–Jensen medium in the dark, yellow after 1 hr. exposure to light. One strain (no. 0735) pigmented yellow only after two exposures of 8 hr. to light.

Group II. Pigmented deep yellow in the dark and in the light on Löwenstein–Jensen and on 7H-10 medium.

Group III. Strains 514, 520, M1208 pigmented deep yellow in the dark and in the light on Löwenstein–Jensen and 7H-10 media. All remaining strains pigmented buff in the dark and in the light.

Group IV and *Mycobacterium tuberculosis.* Pigmented buff in the dark and in the light.

Temperature requirements and rate of growth

All of the atypical mycobacteria grew at 22° within 6 weeks when the cultures were inoculated by the clumped inoculum method. Among strains of *Mycobacterium tuberculosis*, H37Rv did not grow at this temperature; in further experiments with other strains growth occurred in a small proportion of them. Among the 16 group I strains whose cultures were inoculated by the dispersed inoculum method, three did not grow at 22° (nos. A725, M1247, 0523). Considerable differences were found in the rate of growth at 37°, depending upon the inoculum used. Growth was more rapid with a clumped inoculum than with a dispersed inoculum and, with the group III strains, growth occurred 3–4 days earlier when the slopes were inoculated from 6-week cultures than from 2- to 3-week cultures. Whichever inoculum was used, group IV strains grew more rapidly than the remaining strains of atypical mycobacteria, and these grew slightly more rapidly (1–4 days quicker) than *M. tuberculosis* H37Rv.

Growth on blood and nutrient agar

Strains of group I and group II did not grow within 5 days on blood agar and nutrient agar plates. Of the 19 group III strains, 16 did not grow on these media in this period, but 3 strains (nos. P25, 487, 171C) yielded growth within 3 days. All group IV strains grew within 3 days. *Mycobacterium tuberculosis* H37Rv did not grow.

Growth in gelatin stabs

Group I strains, group II strains, *Mycobacterium tuberculosis* H37Rv and those group III strains which did not grow on blood and nutrient agar, did not grow in gelatin stabs. The 3 group III strains, which yielded growth on blood and nutrient agar, grew in gelatin stabs within 2 weeks. These strains formed colonies on the top and a filiform growth in the stab; liquefaction was not observed. All group IV strains grew within 2 weeks, forming colonies on the tops and a filiform growth in the stabs, and did not liquefy the medium.

The nicotinic acid test

All strains of atypical mycobacteria yielded a negative nicotinic acid test, while *Mycobacterium tuberculosis* H37Rv yielded a positive result.

Catalase activity

The results of catalase tests are shown in Table 2. All group I, group II and group IV strains showed catalase activity greater than with *Mycobacterium tuberculosis* H37Rv when grown on drug-free medium and on medium containing isoniazid, as did their isoniazid-resistant variants. Group III strains showed a variable pattern of catalase activity, some strains having activities similar to those of the remaining atypical strains and others yielding a pattern more closely resembling that of *Mycobacterium tuberculosis* H37Rv.

Table 2. *Catalase activity of mycobacteria*

		Catalase activity*		
		Original strain		
Group	Strain no.	Growth on drug free medium	Growth on medium containing highest isoniazid concentration allowing growth	Variant resistant to 50 µg. isoniazid/ml.
I	All 16	+ + +	+ + +	+ + +
II	All 4	+ + +	+ + +	+ + +
III	171C, 487, 513, 514, 520, P7, P25	+ + +	+ + +	+ + +
	160, 225, 470, P23	+ + +	+ +	+ +
	248, 277, 509, 585	+ +	+ +	+ +
	170, 477	+ +	+ +	0
	223, M1208	+ +	+	+
IV	All 3	+ + +	+ + +	+ + +
M. tuberculosis	H37Rv	+ +	0	0

* Catalase activity scored by number of + signs.
Catalase activity more marked than that of strain *Mycobacterium tuberculosis* H37Rv, + + +; equal to that of strain H37Rv, + +; present, but less than that of strain H37Rv, +; no activity, O.

Sensitivity tests

The results of the sensitivity tests are set out in Tables 3 and 4. Classifications as sensitive, doubtfully resistant, and resistant are based on experience with strains of *Mycobacterium tuberculosis*. All of the strains of atypical mycobacteria were resistant to isoniazid and all were resistant to *p*-aminosalicylic acid, with three exceptions (group I, strains nos. 0523, 0735 and 260). All group IV strains were resistant to streptomycin, but about one third of the remainder were sensitive. All of the group I strains were sensitive or doubtfully resistant to cycloserine. Of the 22 group II and group III strains tested, one in each group (nos. 0112, 170) was resistant to cycloserine, as were all of the group IV strains. All of the group I and

Table 3. *Sensitivity of atypical mycobacteria to isoniazid,* p-*aminosalicylic acid and streptomycin*

Group	No. of strains tested	Isoniazid minimal inhibitory concentration (μg./ml.)					*p*-Aminosalicylic acid resistance ratio*					Streptomycin resistance ratio*				
		Resistant			Doubtfully resistant	Sensitive	Resistant			Doubtfully resistant	Sensitive	Resistant			Doubtfully resistant	Sensitive
		>50	50	5	1	0·2	>16	16	8	4	2 or less	>256	(256–8)	8	4	2 or less
I	16	3	7	3	3	0	12	1	0	0	3	0	3	4	4	5
II	4	4	0	0	0	0	2	0	1	1	0	0	0	0	3	1
III	19	12	6	0	1	0	18	0	1	0	0	1	8	2	0	8
IV	3	1	1	0	1	0	3	0	0	0	0	1	2	0	0	0

* Resistance ratio: the minimal inhibitory concentration for the test strain: the minimal inhibitory concentration for the standard sensitive strain H37Rv.

Table 4. *Sensitivity of atypical mycobacteria to cycloserine, thioamide and thiacetazone*

Group	No. of strains tested	Cycloserine resistance ratio*					No. of strains tested	Thioamide resistance ratio*					No. of strains tested	Thiacetazone resistance ratio*		
		Resistant			Doubtfully resistant	Sensitive		Resistant			Doubtfully resistant	Sensitive		Resistant		Sensitive
		>16	16	8	4	2 or less		>16	16	8	4	2 or less		>8	8	4 or less
I	12	0	0	0	3	9	12	0	0	0	0	12	11^{2}	3	0	8
II	3	0	0	1	0	2	3	0	0	0	0	3	2^{3}	2	0	0
III	19	1	0	0	2	16	18^{1}	5	0	5	4	4	17^{4}	16	0	1
IV	3	3	0	0	0	0	3	0	0	2	1	0	1^{5}	1	0	0

* Resistance ratio: the minimal inhibitory concentration for the test strain: the minimal inhibitory concentration for the standard strain of *Mycobacterium tuberculosis.*

[1] Strain no. 170 not tested to thioamide; [2] Strain no. A. 725 not tested to thiacetazone; [3] Strain no. 0112 not tested to thiacetazone; [4] Strains nos. 470, 520 not tested to thiacetazone; [5] Strains nos. 380, 518 not tested to thiacetazone. Strains nos. M1159, M1212, M1246, M1247 (group I) and strain no. M1177 (group II) not tested to any of these three drugs.

group II strains were sensitive to thioamide and the remaining groups showed a variable pattern of sensitivity. Of the 11 group I strains tested, 8 were sensitive to thiacetazone whereas all except one of the remaining strains (no. 514) were resistant. These sensitivity tests were not repeated systematically and, from further experience, a slightly different pattern of resistance, particularly to *p*-aminosalicylic acid and thiacetazone, might have been encountered if several tests had been set up on each strain.

Characteristics of the different groups

Those features of the strains belonging to the four groups which were found to be most consistent and characteristic of the groups, are given below.

Group I. Long, beaded, strongly acid-fast, periodic acid-Schiff positive. Tendency to cord formation. Colonies of the smooth strains (CM type Ia) have central areas of opacity. Colonies of the rough strains (CM type Ib) similar to those of *Mycobacterium tuberculosis*, strain H37Rv. Yellow pigmentation only after exposure to daylight. No growth on blood and nutrient agar at 37° within 3 days, and no growth in gelatin stabs at 22° within 6 weeks. Nicotinic acid test negative. Catalase activity strongly positive even when grown on isoniazid-containing medium. Resistant to isoniazid; sensitive to thioamide; sensitive or doubtfully resistant to cycloserine; often sensitive to thiacetazone.

Group II. Beaded, strongly acid-fast, period acid-Schiff positive organisms of variable length. No tendency to cord formation. Colonies smooth, with entire edges. Pigmented deep yellow when grown in the dark, as well as after exposure to light. No growth on blood and nutrient agar at 37° within 3 days and no growth in gelatin stabs at 22° within 6 weeks. Nicotinic acid negative. Catalase activity strongly positive, even when grown on isoniazid-containing medium. Resistant to isoniazid and thiacetazone; sensitive to thioamide; sensitive or doubtfully resistant to streptomycin.

Group III. Organisms either uniformly shorter than those of *Mycobacterium tuberculosis* H37Rv or pleomorphic. Staining either uniform or beaded. Either all organisms acid-fast or many not acid-fast. No tendency to cord formation (tight cord formed by one strain). Periodic acid-Schiff negative. Colonies of smooth strains (CM type IIIa–IIId) variable in size, with entire edges and uniform structure. (One strain with central area of opacity.) Colonies of rough strains (CM type IIIe) similar to those of *M. tuberculosis* strain H37Rv, but smaller and more variable in size. Colonies of 16 strains pigmented buff, but colonies of the remaining 3 strains pigmented yellow. No growth on blood and nutrient agar at 37° within 3 days, and no growth in gelatin stabs within 6 weeks (3 exceptions). Catalase activity variable, often diminished when grown on isoniazid-containing slopes. Resistant to isoniazid and *p*-aminosalicylic acid; sensitive or doubtfully resistant to cycloserine (1 exception).

Group IV. Bacillary morphology, staining properties (including acid-fastness and periodic acid-Schiff staining), morphology of colonies and pigmentation the same as those of *Mycobacterium tuberculosis*, strain H37Rv. Growth on blood and nutrient agar at 37° within 3 days, and growth in gelatin stabs at 22° within 6 weeks. Nicotinic acid test negative. Catalase activity strongly positive even when grown on isoniazid-containing medium. Resistant to isoniazid, *p*-aminosalicylic acid, streptomycin and cycloserine; resistant or doubtfully resistant to thioamide.

DISCUSSION

Two main problems arise during the course of identifying mycobacteria obtained from material of human origin: (1) the differentiation of *Mycobacterium tuberculosis* from the atypical mycobacteria, and (2) their classification. In these experiments, the most useful method for differentiating *M. tuberculosis* from the atypical mycobacteria was found to be the nicotinic acid test. All of the strains of atypical mycobacteria yielded a negative result, whereas strain *M. tuberculosis* H37Rv gave a positive result. Using the same technique, Gilani & Selkon (1958) found that all of 13 human strains of *M. tuberculosis* yielded a positive nicotinic acid test. Runyon, Selin & Harris (1959), who used a different technique, reported their own findings and those of other workers, and found that the nicotinic acid test was uniformly negative for atypical mycobacteria and uniformly positive for human *M. tuberculosis*. The main disadvantage of the nicotinic acid test is that bovine strains of *M. tuberculosis* yield a negative result, so that strains of mycobacteria which yield such a result must be further characterized, for example by guinea-pig inoculation. Growth at 22° was not reliable as a means of differentiating *M. tuberculosis* from atypical mycobacteria since with the clumped inoculum occasional strains of *M. tuberculosis* yielded growth, and with the dispersed inoculum some of the atypical mycobacteria tested did not grow. Growth at 25° may be more satisfactory (Marks & Trollope, 1960). The following characteristics were also of value in identifying atypical strains: long, beaded organisms; weakly acid-fast; lack of cord formation; bacillary pleomorphism; periodic acid-Schiff positivity; smoothness of colonies; yellow pigmentation of colonies; growth on blood and nutrient agar within 3 days; growth in gelatin stabs cultures; high catalase activity on medium containing isoniazid and a characteristic sensitivity pattern. No one of these characteristics was invariably shown by all of the atypical strains. However, when several of these characters were examined, all of the atypical strains were distinguishable from *M. tuberculosis*.

The following characteristics were found to be of most value in classifying the atypical mycobacteria into the groups proposed by Runyon (1959*a*). *Group I:* formation of yellow pigment after exposure to light for 1 hr. was described as the essential characteristic of strains in this group. However, one of our strains required a longer period of exposure, but it was still included with other strains in this group because of the similarity of their behaviour in other respects. Strains with smooth colonies (CM type Ia) could usually be classified by their colonial morphology, but rough colonies (CM type Ib) could be confused with those of *Mycobacterium tuberculosis* and of group IV strains. Positive periodic acid-Schiff staining was an invariable feature in this group but was also found in group II strains. *Group II:* formation of yellow or orange pigment in the dark (Runyon, 1959*a*) was invariably found with all strains, but also occurred with 3 of the group III strains. Positive periodic acid-Schiff staining was shown by all strains in this group, and also by those in group I. Colonial morphology was also of value. *Group III:* among the 19 strains classified in this group, 3 yielded yellow pigmentation both in the dark and in the light, and on both Löwenstein-Jensen and 7H-10 media. Nevertheless, they resembled the remaining strains in this group in other characteristics and should be retained in it. A further 3 strains grew on nutrient agar and Löwenstein–Jensen medium within

3 days and they also grew in gelatin stab cultures. It would therefore be reasonable to transfer these 3 strains to group IV. With the remaining strains classified in group III, diversity of all of the characteristics studied was noted, and this group cannot be considered as homogeneous. *Group IV:* Runyon did not describe in detail the specific conditions necessary to identify these strains as rapidly-growing ones. In the present work the speed of growth of the strains in all of the groups on Löwenstein–Jensen medium was dependent on the size and type of the inoculum, so that it was not always easy to distinguish group IV strains from the remainder. The ability to grow on blood and nutrient agar plates within 3 days was found only with group IV strains and with the three group III strains mentioned above. The superiority of the test on blood or nutrient agar may be due not only to differences in growth rate, but also the more satisfactory nature of these media for group IV strains. The ability to grow in gelatin stab cultures within 6 weeks was also of value for characterizing these strains. Further experience with 32 strains of saprophytic mycobacteria confirms the value of blood and nutrient agar and of gelatin stab cultures in their identification as group IV strains.

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Isolation and Classification of a New Series of Azotobacter Bacteriophages

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SUMMARY

Successful isolation of azotobacter phages from soil samples was accomplished by using a modified Burk's nitrogen-free medium with sucrose as the carbon source. The natural azotobacter flora in the soil samples served as the enrichment strains and the medium was not further enriched by the addition of laboratory cultures of the bacteria. Phage titres as high as $5{\cdot}5 \times 10^9$ plaque-forming units/ml. were obtained when the indicator strain for phage assays was *Azotobacter vinelandii* O. On the basis of plaque morphology, nine phage isolates were obtained and purified by standard techniques. The plaques formed by the phages consisted of a central clear area surrounded by a halo and ranged from 1 to 7 mm. in diameter. Antiphage sera were produced in rabbits against a previously isolated phage and the new isolates; on the basis of cross-neutralization experiments with homologous and heterologous antisera, the 10 phages were placed into four major serological groups. Groups I and II contained four phages each, and groups III and IV contained one phage each. The degree of serological relatedness among the phages within groups I and II was investigated. A survey of 48 azotobacter strains showed that 11 out of 12 *A. vinelandii* strains and 14 out of 25 *A. chroococcum* strains showed plaque formation by one or more of the phages. Strains of *A. agilis*, *A. macrocytogenes*, *A. insigne* and *A. indicus* were not lysed by the phages. The value of the present series of phages in the classification of the genus *Azotobacter* was discussed.

INTRODUCTION

A bacteriophage for certain strains of the genus *Azotobacter* designated *A. vinelandii* was originally isolated and characterized by Monsour (1954), Monsour, Wyss & Kellogg (1955) and Kellogg (1957). Since plaques were not observed with the non-pigmented strains of *A. agilis* or strains of *A. chroococcum* (Monsour *et al.* 1955), the securing of additional phages appeared desirable since they would serve as a supplementary tool in the study of ecology and mutations in these organisms and would be an aid in the species designation of this genus. In addition, most of our present information regarding the bacteriophages has resulted from investigations mainly on the coli phages. Further characterization of additional bacteriophages and phage host systems would aid in confirming and generalizing phenomena already postulated, or might lead to new developments. The azotobacters are especially suited to such an investigation because of their many unique characteristics. They are larger than the average bacteria, are capable of abundant growth in a simple medium free from combined nitrogen, possess a high respiratory activity and to a certain extent are genetically stable organisms. The present investigation describes the isolation and classification of a new series of azotobacter phages.

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METHODS

Strains. Most of the work was accomplished by using *Azotobacter vinelandii* O, a strain originally obtained from the University of Wisconsin. This strain was subcultured every 4 days on Burk's modified nitrogen-free medium and the colonial morphology of the strain was routinely examined by visual inspection of individual clones, with the use of oblique illumination. On several occasions a smooth clone with the least amount of opaqueness was selected and purified by dilution and plating. Incubation was at 33° for 24–48 hr. Other strains of azotobacter used included the following: *A. vinelandii*, American Type Culture Collection (hereafter referred to as ATCC) strains 478, 7484, 7487, 7489, 7492, 7496, 9046, 9047, 9104, 12837; University of Texas Stock Culture Collection (hereafter referred to as UT) strain K; *A. agilis*, ATCC strains 7494, 9040, 9042, 9570, 12838; UT strain M; *A. chroococcum*, ATCC strains 480, 4412, 7486, 7488, 7490, 7491, 7493, 7497, 7498, 7499, 9043, 9044, 9045, 9048, 9049, 9051, 9335, 9544, 12981; UT strains A, B, C, D, E, F, J, S; *A. indicus*, ATCC strains 9037, 9038, 9039, 9540; *A. macrocytogenes*, ATCC strains 12334, 12335, 12336; *A. insigne* strains 8 and 2, received from Dr Vagn Jensen (University of Lund, Lund, Sweden).

Media. The following media were used:

Burk's modified nitrogen-free medium (Kellogg, 1957). The medium contained (g./l.): Na_2HPO_4, 0·189; KH_2PO_4, 0·011; $MgSO_4.7H_2O$, 0·200; $FeSO_4.7H_2O$, 0·006; MoO_3, 0·0005; $CaSO_4.2H_2O$, 0·02; $SrCl_2.6H_2O$, 0·01; NaCl, 0·01; $NaHCO_3$, 0·05. De-ionized water was used in the preparation of this medium. The medium was at pH 7·8 before autoclaving (18 lb./sq.in. for 20 min.). When sucrose was added as the carbon source, it was autoclaved (18 lb./sq.in. for 20 min.) in 50% (w/v) solutions and added to the medium to give a final concentration of 0·5% (w/v). Hereafter, the term 'Nf medium' is used instead of 'nitrogen-free medium'.

ATCC media. The base medium contained (g./l.): K_2HPO_4, 1·0 g.; $MgSO_4.7H_2O_2$, 0·2; NaCl, 0·2; $FeSO_4.7H_2O$, trace; soil extract, 100 ml.; tap water, 900 ml. The soil extract was prepared by adding 77 g. of a commercial African violet potting soil and 0·2 g. Na_2CO_3 to 200 ml. distilled water. The mixture was autoclaved (18 lb./sq.in. for 1 hr.) and filtered through a Buchner funnel. The four media prepared from the above stock were: ATCC-1, adjusted to pH 8·3, 2% (w/v) mannitol added, autoclaved, final pH 7·6; ATCC-2, adjusted to pH 6·0, 2% (w/v) mannitol added, autoclaved; ATCC-3, adjusted to pH 6·0, 2% (w/v) glucose added, autoclaved, ATCC-4, adjusted to pH 7·6, autoclaved, sterile glucose to a final concentration 1·0% (w/v). All autoclaving was at 18 lb./sq.in. for 20 min.

Phages. Ten phages were used in these studies. One phage, designated 'Original' or 'A-11' for these studies, was reported by Monsour *et al.* 1955; the other nine phages were new isolates made in this laboratory.

Isolation of phage from soil. One-litre flasks containing 50 ml. Nf medium with 0·5 % (w/v) sucrose as carbon source were seeded with 1·0 g. soil and incubated on a shaker at 33° for 4 days. At this time, the contents of the flask were clarified by centrifugation, passed through a membrane filter (Millipore, 047 mm.) and assayed for phage by the double agar layer technique. The indicator strain for the phage assays was *Azotobacter vinelandii* O.

Phage assay. Phage dilutions were made in Nf medium minus carbon source, and plaque counts were made by the overlay method (Adams, 1950) modified by Kellogg (1957) for use with azotobacter phage. Approximately 35 ml. Nf medium containing 1·8 % agar (w/v) were used for the base layer, and 2·0 ml. containing 0·5 % (w/v) agar were used for the semisolid agar layer that contained the mixture of the suspension of bacteria and phage. Unless otherwise stated, both layers of media contained sucrose at a final concentration of 0·5 % (w/v). Incubation was at 33° for 24 hr. or until satisfactory plaque formation was observed (the plates were not inverted).

Preparation of indicator strain. The strain used for the background growth was grown in 15 ml. volumes of Nf medium containing 0·5 % (w/v) sucrose and incubated at 33° on a shaker for 12–18 hr. After growth, the culture was centrifuged at room temperature, the organisms washed, resuspended in de-ionized water and adjusted to a turbidity reading of 35 units on a Klett–Summerson photoelectric colorimeter (blue filter). Unless otherwise stated, *Azotobacter vinelandii* O was used as the indicator strain.

Propagation of phage. For the preparation of stock phage, phages were grown using *Azotobacter vinelandii* O as the propagating strain. Organisms were grown in litre flasks containing 100 ml. Nf medium + 0·5 % (w/v) sucrose. Incubation was on a shaker at 33° until a turbidity reading of 35 units on the Klett–Summerson photoelectric colorimeter (blue filter) was obtained. This turbidity represented 3×10^7 viable organisms/ml. Phages were added to the organisms at a 1:1 ratio of phage:organisms, and the organism + phage mixture allowed to remain static at 33° for 30 min. at which time it was placed on a shaker at the same temperature for 7–13 hr.

A second method for obtaining phage stocks was used for two phages (A-24, A-41). These phages were assayed by the overlay method and the plate from the highest dilution which showed almost complete lysis was selected. The soft agar layer was scraped from the plate and placed in 7·5 ml. Nf media. After overnight extraction at 4°, the agar was removed by filtration through cheesecloth, and the bacterial debris sedimented at low speed in a centrifuge. The supernatant fluid was assayed, and the process repeated until no further increase in phage titre was observed. At this time, a larger quantity of phage stock was obtained by preparing 10–20 plates at the phage dilution which gave complete to partial lysis. Following propagation, phage preparations were stored overnight at 4° to allow debris to settle out. The supernatant fluids were decanted, centrifuged at 700 rev./min. for 30 min. and passed through a membrane filter (Millipore, 047 mm). The phage filtrates were stored at 4°.

Preparation and assay of antiphage sera. For the initial series of immunizations, rabbits were immunized subcutaneously with 5 ml. phage filtrate at 5-day intervals until a series of six injections had been administered. The animals were then bled at either 6 or 15 days following the last injection. Booster immunizations consisted of two 5·0 ml. subcutaneous injections given 5 days apart and for the majority of the phages were administered 1 month following the last injection of the initial series. All sera were collected in the usual manner and stored at −20° without preservative.

The rates of inactivation of the various azotobacter phages by their homologous and heterologous antisera were determined by the following procedures. All dilutions were made in Nf medium. Stock phages were diluted to contain approximately 10^5 to 10^6 plaque-forming units/ml. (the term 'plaque-forming units' is hereafter referred to as 'PFU'). The sera were diluted to a concentration determined by preliminary tests which permitted approximately 1–10 % survival of the PFU after sera and phage dilutions were mixed and incubated at 37° for 5–10 min. Phage and serum dilutions were preheated to 37° in a water bath. At time zero, 5·0 ml. phage dilution were added to 5·0 ml. serum dilution, and at specified time intervals 0·1 ml. samples were removed and mixed with 9·9 ml. Nf medium. The initial 1/100 dilution was sufficient to stop the neutralizing action of the antisera. Dilutions within the range required were made to detect phage survival by the plaque count method. Plating was done in duplicate by the overlay method in the usual manner. A phage control containing 5·0 ml. Nf medium instead of immune sera was run simultaneously and served as a basis for determining % survival of phage. The results of the assays of surviving phage of each neutralization were plotted on a logarithmic scale against time on a linear scale. A straight line was drawn through the points, and the K value determined (Adams, 1959).

Neutralization technique. Cross-neutralization assays with the various azotobacter phages and antiphage sera were carried out in accordance with the following procedures. All antisera were used at a final dilution of 1/200 except antiserum A-41, which was used at a final dilution of 1/100. Three ten-fold serial dilutions were used for each phage and the dilutions were calculated so that the middle dilution would provide approximately 100 PFU/ml. in the final dilution. Dilutions of phage and antisera were made in Nf medium minus carbon source. Equal quantities of the various phage dilutions and antisera dilutions were mixed and incubated in a water bath at 37° for 30 min. Two controls were run simultaneously: one contained Nf medium instead of antiserum, the second contained normal rabbit serum at the same dilution as the immune sera. Following incubation, the assay tubes were placed in an ice water bath, and samples were plated by the double agar layer method in the usual way. Plaques were counted and a comparison made between the controls and immune sera.

Host specificity assays. A modification of the overlay technique was used in these experiments. Nf medium was used for growth of all strains with the following exceptions: *Azotobacter macrocytogenes* strains 12334, 12335, 12336, medium ATCC-2; *A. indicus* strains 9037, 9038, 9039, 9450, medium ATCC-3; *A. chroococcum* strain 9048, medium ATCC-4, strain 7499, medium ATCC-1; *A. agilis* strains 9042, 9570, medium ATCC-4. Petri plates measuring 14 cm. diam. × 2 cm. were used. Approximately 100–150 ml. agar medium was used for the basal layer, and 10 ml. was used for the soft agar layer which contained 0·5 ml. of a bacterial suspension. Young broth cultures of azotobacter strains were centrifuged, washed, resuspended in water and standardized to a reading of 40 units on the Klett–Summerson photoelectric colorimeter (blue filter) with the exception of the *A. macrocytogenes* strains which were standardized to 50 units. The plates were subdivided into 20–25 squares, and dilutions of the phages ranging from 1/10 to 1/100,000 were pipetted in 0·02 ml. amounts on to the top layer of agar. Following absorption of the phage suspensions, the plates were incubated at 33° for 18–24 hr.; the time depended upon the growth rate of the various strains.

RESULTS

Isolation of phage from soil

Preliminary experiments indicated that the natural azotobacter flora present in the soil samples under investigation would serve as the enrichment strains and that addition of laboratory cultures of the bacteria to the culture medium was not necessary. Soil samples were obtained from various geographical localities, and phages were isolated according to the procedures described in the previous section. The results of the isolation experiments are presented in Table 1.

Table 1. *Results of azotobacter phage isolations from six different soil samples*

Soil source	Flask no.	Phage assay PFU/ml.	No. different phages isolated	Present phage designation
Maryland	MD-1	$1{\cdot}4 \times 10^9$	1	A-22
	MD-2	$5{\cdot}5 \times 10^9$		
Ohio	OH-1	9×10^8	2	A-12
	OH-2	1×10^7		A-24
Texas	SA	$1{\cdot}2 \times 10^7$	2	A-14
				A-23
Texas	VA	$3{\cdot}7 \times 10^7$	2	A-13
				A-21
Texas	9	1×10^5	1	A-41
Texas	FB	1×10^8	1	A-31

By these procedures, phage titres ranging from 10^5 to 10^9 PFU/ml. were obtained, and on the basis of plaque morphology, nine different phages were isolated and purified by further single plaque selection. From three of the soil samples two distinct phage isolates were made, whereas the remaining samples appeared to have homogeneous plaques.

Plaque morphology

In the selection of plaques during the purification of azotobacter phages by single-plaque selection, an attempt was made to select plaques of extreme size, for example, minute or large; plaques with small clear areas and wide turbid halos; or plaques with large clear areas and small halos. Photographs of the plaques produced by the azotobacter phages are shown in Pl. 1, figs. A–I, and a description of the plaque size is presented in Table 2.

The host strain for these studies was *Azotobacter vinelandii* O, and the plating conditions were the same as those previously described. The plaques consisted of a central clear area surrounded by a halo and ranged in size from 1 to 7 mm. in diameter. This halo was present even in very small plaques in which the central

Table 2. *Plaque size of azotobacter phages with* Azotobacter vinelandii O *as the indicator strain*

Phage	Plaque diameter (mm.)	Halo diameter (mm.)	Diameter of clear area of lysis (mm.)
A-11	1–4	0·5–1·5	0·5
A-12	1	< 1	< 1
A-13	4	1·5	1
A-14	1–2	0·5	1
A-21	6–7	1–1·5	3·5–4
A-22	3–5	1–1·5	2
A-23	3–5	1	2·5–3
A-24	3	1	1
A-31	4	1	2
A-41	3–4	1	0·5

clear area was not very apparent. The plaques observed with these phages were of four types: (1) minute, represented by A-12 and A-14; (2) small central clear area with wide halo, represented by A-13 and A-11; (3) large central clear area with narrow halo, represented by A-21, A-22, A-23 and A-31; (4) large central clear area with irregular or 'frayed' halo, represented by A-41. At early readings this plaque no. 4 gave the appearance of not having a halo; however, when the plates were refrigerated overnight, a more pronounced halo was observed. This observation was also true for the minute plaques of phages A-12 and A-14.

Propagation of phages

Although optimum conditions for maximum phage titre were not determined, high-titre phage stocks were prepared from the original phage (A-11) and eight of the newly isolated phages according to the methods described in the previous section. The results of a propagation experiment are presented in Table 3.

Table 3. *Preparation of phage stocks with* Azotobacter vinelandii O *as the propagating strain*

Phage	PFU/ml.	Phage added (ml.)	Azotobacters* added (ml.)	PFU/ml. in flask before incubation	Time on shaker (hr.)	PFU/ml. after incubation
A-11	$1{\cdot}7\times10^{9}$	2	98	$3{\cdot}4\times10^{7}$	13	1×10^{9}
A-12	$1{\cdot}3\times10^{9}$	2	98	3×10^{7}	13	8×10^{8}
A-13	$1{\cdot}3\times10^{9}$	2	98	3×10^{7}	7	$1{\cdot}5\times10^{9}$
A-14	$3{\cdot}2\times10^{9}$	1	99	3×10^{7}	7	1×10^{9}
A-21	$1{\cdot}5\times10^{9}$	3	97	3×10^{7}	7	$1{\cdot}3\times10^{9}$
A-22	$1{\cdot}6\times10^{10}$	0·2	99·8	3×10^{7}	13	5×10^{10}
A-23	1×10^{9}	3	97	3×10^{7}	7	$1{\cdot}3\times10^{9}$
A-31	6×10^{9}	1	99	6×10^{7}	13	4×10^{9}

* 3×10^{7} viable organisms/ml.

The *Azotobacter vinelandii* strain O was used at a concentration of 3×10^{7} viable organisms/ml. and the ratio phage:organisms at the start of the experiment was approximately 1:1. For the majority of the phages, final titre of 10^{9} PFU/ml. was achieved. Four of the phage preparations showed complete lysis of the bacterial culture after incubation on the shaker for *c.* 7 hr.; for the remaining preparations an arbitrary incubation time of 13 hr. was selected. With the majority of the azotobacter phages a considerable amount of bacterial debris remained after lysis.

Two of the azotobacter phages (A-24, A-41) were not successfully propagated according to the above methods; therefore a modification of the agar layer technique was used. By these procedures, the highest titres obtained for phages A-24 and A-41 were 5×10^8 and 5×10^7 PFU/ml., respectively. All phage preparations appeared stable following Millipore filtration and storage at 4° with the exception of phage A-11; however, non-filtered preparations of this phage appeared to be stable.

Table 4. *Cross-neutralization by representative antisera against various azotobacter phages*

Antisera	Phages									
	A-11	A-12	A-13	A-14	A-21	A-22	A-23	A-24	A-31	A-41
A-11	+	+	+	+	−	−	−	−	−	−
A-12	+	+	+	+	−	−	−	−	−	−
A-13	+	+	+	+	−	−	−	−	−	−
A-14	+	+	+	+	−	−	−	−	−	−
A-21	−	−	−	−	+	+	+	+	−	−
A-22	−	−	−	−	+	+	+	+	−	−
A-23	−	−	−	−	+	+	+	+	−	−
A-31	−	−	−	−	−	−	−	−	+	−
A-41	−	−	−	−	−	−	−	−	−	+
Normal serum	−	−	−	−	−	−	−	−	−	−

+ = neutralization; − = no neutralization. All antisera and the normal serum were used at final dilution 1/200, except antiserum A-41 which was used at final dilution 1/100.

Serological classification

The cross-neutralization data (Table 4) indicated that the phages formed four main serological groups; groups one and two contained four phages each, and groups three and four contained one phage each. Although antiserum against phage A-24 was not prepared, this phage was neutralized by the available group two antisera. On the basis of these data, the following serological groups for the azotobacter phages are proposed and will be referred to in subsequent discussions: group I (phages A-11, A-12, A-13, A-14); group II (phages A-21, A-22, A-23, A-24); group III (phage A-31); group IV (phage A-41).

Kinetics of phage neutralization

The neutralization rate constants of the various phages by their respective homologous antisera are presented in Table 5. Since the K value is a characteristic of the particular lot of serum used, the serum data have been included for reference purposes. The phages were neutralized by dilutions of homologous antisera ranging from 1/500 to 1/5000, and the K values ranged from 18 to 1917. As reported for other phage systems, the azotobacter phages differed greatly with respect to their rates of reaction with homologous antisera. As a group, the phages belonging to

Table 5. *Neutralization rate constants* (*K*) *of azotobacter phages with homologous antisera*

Phage	Final serum dilution	Serum date	PFU/ml.	K
A-14	1/5000	8/26/58	$7{\cdot}8\times10^6$	1917
A-21	1/5000	8/26/58	$3{\cdot}3\times10^6$	1642
A-22	1/5000	2/3/58*	$8{\cdot}9\times10^6$	1534
A-23	1/2000	8/26/58	$3{\cdot}3\times10^6$	742
A-31	1/1000	11/4/57	$8{\cdot}0\times10^6$	575
A-13	1/2000	8/26/58	$3{\cdot}7\times10^6$	460
A-11	1/1000	10/21/57*	$1{\cdot}7\times10^6$	329
A-12	1/200	8/26/58	$5{\cdot}5\times10^5$	115
A-41	1/150	9/27/58	$1{\cdot}3\times10^5$	18

* Pooled sera.

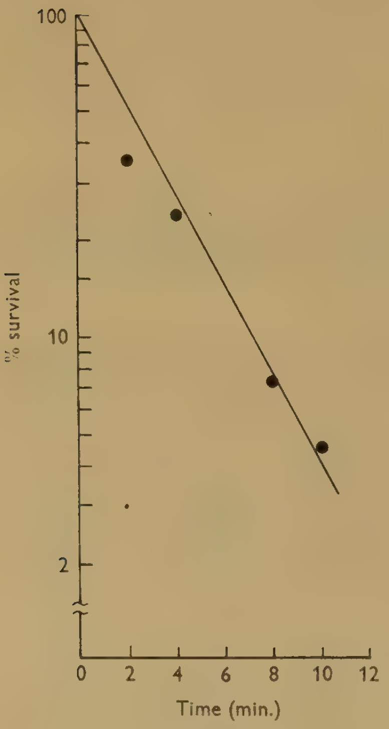

Fig. 1

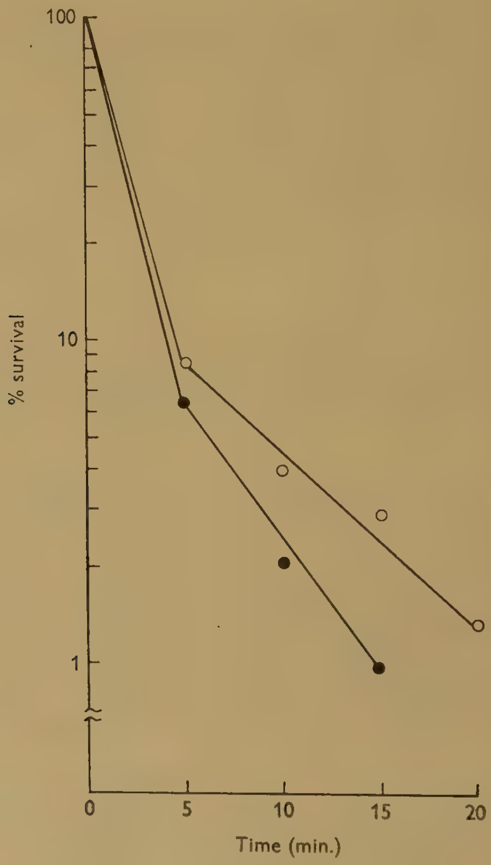

Fig. 2

Fig. 1. Rate of inactivation of phage A-11 by homologous antiserum (diluted 1/1000).

Fig. 2. Rates of inactivation of phage A-12 by homologous antiserum. The antiserum was diluted 1/200. ○, Expt. 1; ●, Expt. 2.

serological group II were among the better antigens, since their antisera showed relatively high K values. The high K value for serum A-14 indicated phage A-14 acted as a much better immunizing antigen than the other phages (A-11, A-12, A-13) of serological group I. The low K value observed with serum A-41 may be attributed to the relatively low concentration of phage used in the immunizing antigen.

The neutralization curves from which the K values were calculated are presented in Figs. 1–7. The values for sera A-21, A-22 and A-23 were obtained from the curves shown for the homologous neutralizations shown in Figs. 5–7.

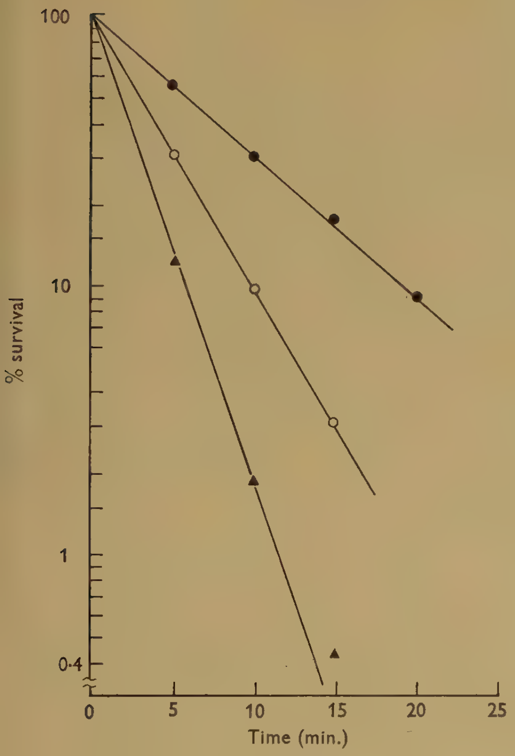

Fig. 3

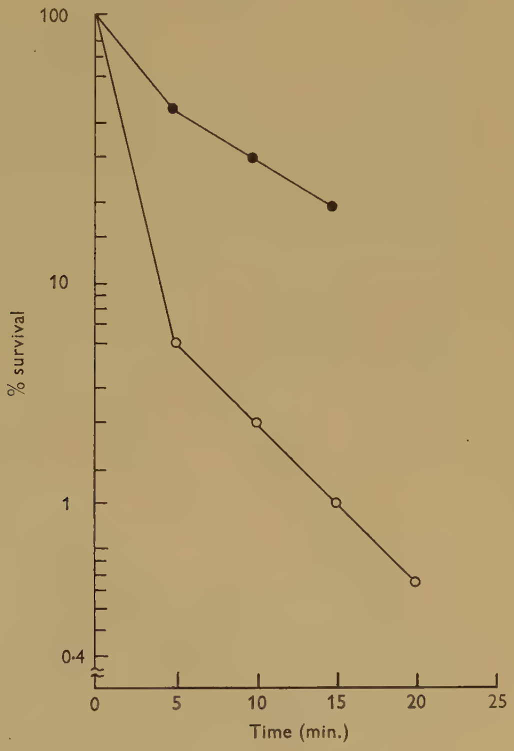

Fig. 4

Fig. 3. Rates of inactivation of phages A-13, A-14 and A-41 by homologous antisera. The antisera were diluted 1/2000 for A-13, 1/5000 for A-14 and 1/150 for A-41. ○, Phage, A-13; ▲, phage A-14; ●, phage A-41.

Fig. 4. Rates of inactivation of phage A-31 by homologous antisera. For Expt. 1, the phage was preparation 3/17/59 and the antiserum was lot number 1/11/58. The antiserum was diluted 1/2000. For Expt. 2, the phage was preparation 6/23/58 and the antiserum was number 4 of 11/5/57. The antiserum was diluted 1/1000. ●, Serum 1/11/58; ○, serum 11/5/57.

With phages A-12 and A-31 neutralization by homologous antisera did not follow first-order reactions. Figure 2 shows two separate neutralization curves obtained for phage A-12 with its homologous antiserum. Neutralization of phage proceeded rapidly to *c.* 96–98 % inactivation of phage and then slowed down rather abruptly. Figure 4 shows that inactivation of phage A-31 behaved similarly. With phage A-31 both the pooled sera (date 1/11/58) and the individual serum from rabbit 4 (date 11/5/57) showed this peculiarity. Also, the use of a different phage preparation in the experiment did not alter the shape of the curve. It should be noted that for these two phage serum systems the initial slopes of the neutralization curves were used to calculate K values to serve as an estimate of the relative neutralizing potencies of the sera.

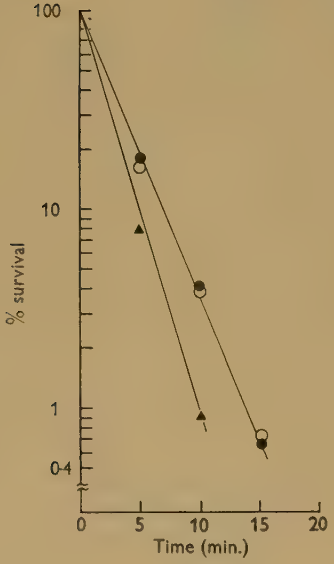

Fig. 5

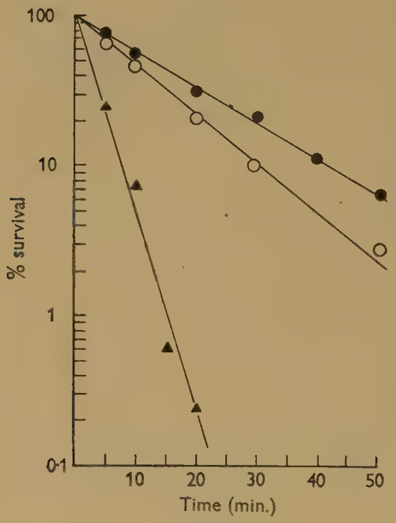

Fig. 6

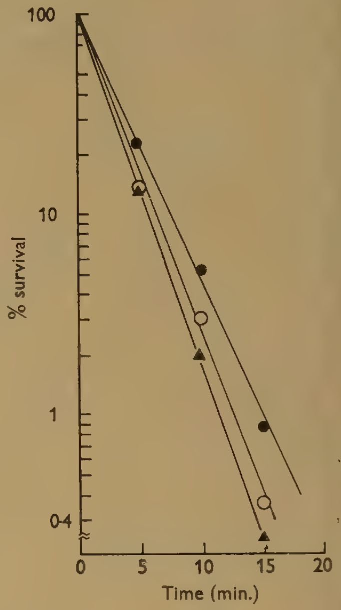

Fig.7

Fig. 5. Rates of inactivation of phages A-21, A-22 and A-23 by A-21 antiserum. The serum was diluted 1/5000 for phages A-21 and A-23 and 1/1000 for phage A-22. ●, Phage A-21; ▲, phage A-22; ○, phage A-23.

Fig. 6. Rates of inactivation of phages A-21, A-22 and A-23 by A-22 antiserum. The antiserum was diluted 1/5000 for the homologous phage and 1/3000 for the heterologous phages. ●, Phage A-21; ▲, phage A-22; ○, phage A-23.

Fig. 7. Rates of inactivation of phages A-21, A-22 and A-23 by A-23 antiserum. The antiserum was diluted 1/2000 for the homologous phage and for phage A-21, and 1/300 for phage A-22.

In order to determine the degree of relatedness among the phages of serological group II, neutralization curves were determined for three of the phages using homologous and heterologous antisera (Figs. 5–7). The K values for the homologous and reciprocal neutralizations are presented in Table 6. The rate of neutralization was greater with homologous phage and antiserum than with the heterologous system, except with antiserum 21 where the neutralization rates for phages A-21 and A-23 were the same. These data indicated that antigenically phages A-21 and A-23 were either very closely related or identical. This conclusion was substantiated with antisera A-22 and A-23. In addition, these data showed that the degree of relatedness of phage A-22 to the other two phages was more distant than the relationship between phages A-21 and A-23.

Table 6. *Neutralization rate constants* (*K*) *of representative group II antisera* against group II phages*

Serum	A-21	A-22	A-23
	Neutralization rate constants (K)		
Anti A-21	1642	460	1642
Anti A-22	173	1534	230
Anti A-23	614	26	742

* 8/26/58 sera.

Preliminary investigations have been conducted on the degree of relatedness among the serological group I phages. Kinetic curves of neutralization using heterologous antiphage sera were not determined for these phage + serum systems; instead, an arbitrary end-point was used, namely the % of the initial phage population which survived after contact with serum at a stated concentration and for a specified time. The procedures were essentially the same as those described for the kinetic studies of phage neutralization except that only one time period was used to assay for phage survival. The time of assay varied with the antisera and was as follows: 5 min. for serum A-12, 10 min. for serum A-13, 6 min. for serum A-14. The final dilutions of sera were 1/200 for A-12, 1/2000 for A-13 and 1/5000 for A-14. All phages were diluted to contain approximately 10^5 PFU/ml.

Table 7. *Neutralization of serological group I phages by homologous and heterologous antisera*

Serum	A-12	A-13	A-14
	% survival of phages		
Anti A-12	5	17	3
Anti A-13	75	41	57
Anti A-14	14	39	14

Table 7 shows the % survival of serological group I phages after neutralization by homologous and heterologous antisera. With serum A-12, % survival was essentially the same for phages A-12 and A-14 and greater for phage A-13. The results obtained with serum A-14 were similar to those observed with serum A-12. Serum A-13 inactivated its homologous phage more rapidly than either of the heterologous phages. These data indicated a close antigenic relationship between phages A-12 and A-14. In other experiments, not shown here, serum A-13 neutralized phages A-11 and A-13 at approximately the same rate, indicating a close relationship between these two phages. With phage A-11 it was important to use a newly produced phage stock. Cell-free filtrates of this phage lost their infectivity rapidly upon storage and older preparations sometimes showed anomalous neutralization patterns.

Host specificity assays

Host specificity assays were undertaken for the purpose of defining further the identity of the phages and also to ascertain the range of host infectiveness. Two of the phages, A-21 and A-23, were shown to be either identical or closely related by serological methods and it was of interest to compare their host-range specificity reactions. These data are presented in Table 8 and it may be observed that both phages gave identical results. Since it appeared that these two phages were identical, further studies on host-range specificity included only one of these phages, namely A-21.

Table 8. *Comparison of host-specificity reactions for phages* A-21 *and* A-23

	Phages			Phages	
Strains	A-21	A-23	Strains	A-21	A-23
A. vinelandii	lytic activity		*A. chroococcum*	lytic activity	
0	5	5†	A	2*	2*
478	5	5	S	5	4
7487	1*	1*	F	—	—
7489	2*	2*	J	—	—
7492	2*	2*	E	5	5
9046	3*	3*	B	—	—
9047	1*	2*	9335	5	5
12837	3	3	9544	4	4
7496	2*	2*	12981	—	—
9104	5	5			
K	4	4			

† Number 5 means lytic action at $1/10^5$ dilution, etc.
* Indicates plaques were not observed. — indicates no lytic action.

Table 9 shows the lytic action of eight phages for 12 strains designated *Azotobacter vinelandii*. All strains, except 7484, were lysed by one or more of the phages. The strain not lysed by the phages appeared to be a typical member of this species. The phages classified as serological group I (A-11 to A-14), showed identical

Table 9. *Lytic action of phages for* Azotobacter vinelandii *strains*

A. vinelandii strains	Phages							
	A-11	A-12	A-13	A-14	A-21	A-22	A-31	A-41
0	5†	5	5	5	5	5	5	5
478	5	5	5	5	5	5	5	5
7487	2	5	5	4	1*	—	2*	1*
7489	3	4	5	4	2*	—	2*	1*
7492	3	3	5	5	2*	—	2*	1*
9046	2*	2*	5*	2*	3*	2*	4*	5
9047	4	3	5	5	1*	—	2*	1*
12837	2*	2*	2*	5*	3	5	2	—
7496	4	3*	5	3	2*	1*	3*	2*
9104	5	3	4	5	5	5	5	5
K	5	2*	5	4	4	5	5	5
7484	—	—	—	—	—	—	—	—

† Number 5 means lytic action at $1/10^5$ dilution, etc.
* Indicates plaques were not observed. — indicates no lytic action.

host range specificity with the exception of phage A-12 which did not form plaques on strains K and 7496. The phages of serological group II, A-21 and A-22, showed identical host range specificity. The lack of lytic action of phage A-22 on several strains was not considered a major difference, since plaque formation with phage A-21 was not observed on the same strains. In addition, the phages of serological group I appeared to be more specific for the *A. vinelandii* strains than the phages of serological group II. One strain, 12837, was not lysed by any phage of group I but by both members of group II. Phage A-31, the only member of serological group III, showed plaque formation on the same five strains identified with serological group II phages. Phage 41, the only member of serological group IV, differed from the other three groups, and especially from group III, by formation of plaques on strain 9046 and its lack of lytic action on strain 12837. It may be noted that phage A-41 was the only phage to show plaque formation on strain 9046.

Table 10. *Lytic action of phages for* Azotobacter chroococcum *strains*

A. chroococcum strains	Phages							
	A-11	A-12	A-13	A-14	A-21	A-22	A-31	A-41
9335	4†	5	5	5	5	2	5	1
7493	5	5	5	5	5	4	5	2
9043	5	5	5	5	3	3	3*	1*
7491	1*	3	5	–	5	4	1	1
9544	2*	2*	3*	2*	4	5	5	5
7486	3*	4	5	2*	5	5	4	3
7490	1*	2*	4	5	4	4	5	4
9045	2*	4	5	±	5	4	4	4
4412	4	4	5	4	5	5	5	1
7499	±	±	5	1*	–	–	5	–
9049	–	–	–	–	–	–	2*	4
7498	–	–	–	–	–	–	2*	–
7488	–	2*	3*	2*	2*	–	4*	–
A	1*	–	2*	1*	2*	2*	2*	–
S	–	–	–	–	5	1	–	5
F	–	–	–	–	–	–	2*	5
J	–	–	±	–	–	–	3*	–
E	4	4	5	5	5	5	2*	±
B	–	–	±	–	–	2*	–	±
C	–	–	±	–	–	2*	–	±

† Number 4 means lytic action at $1/10^4$ dilution, etc.

* Indicates plaques were not observed. ± means questionable lytic action. – indicates no lytic action. Strains 480, 7497, 9048, 9051 and 12981 showed negative reactions with all phages; strain 9044 was not tested.

Table 10 shows the lytic action of eight phages for 25 strains of *Azotobacter chroococcum*. Five of these strains, listed in the notes beneath Table 10, showed negative reactions with all phages. Six additional strains showed non-specific lysis; that is, plaque formation was not observed. With the *A. chroococcum* strains, the problem of rapid growth and abundant slime production was encountered and plaque formation might have been missed. For example, strain F did not show plaque formation after incubation for 20 hr. at 33°. However, when the plates remained at room temperature during the next 12 hr., plaque formation was observed at *c.* 28 hr., but plaques were covered over by the growth of organism and slime formation at *c.* 32 hr.

These data show that *c.* 50 % of the *Azotobacter chroococcum* strains tested were sensitive to one or more of the phages. The phages A-21 and A-22 (serological group II) had identical host range specificity. Phage A-21 appeared to show greater virulence for strain S than did phage A-22; therefore strains could be used to distinguish between or to identify these phages. Whereas the phages of serological groups II and III showed identical host-range specificity with the *A. vinelandii* strains, a few differences were noted among the *A. chroococcum* strains, namely strains 9043 and 7499. Phage A-41 (group IV) was the only phage to show plaque formation with strains F and 9049. The phages of serological group I showed a few differences. The lack of observed plaque formation with these phages could be due to a combination of poor plaque morphology shown by these phages and the slimy, rapid growth of the strains. Further testing is warranted to determine whether these differences are valid.

A summary of the host-range specificity reactions is presented in Table 11. Only those lytic reactions where plaque formation was observed were included. None of the phages lysed any of the six strains designated *Azotobacter agilis*, the three strains of *A. macrocytogenes* or the two strains of *A. insigne*. Phage sensitivity of the *A. indicus* strains was difficult to assess because of their slow growth and copious formation of gummy material. However, preliminary studies indicated that these strains were not lysed by any of the phages.

Table 11. *Summary of host-specificity reactions of azotobacter phages*

	No. of strains	Number of strains lysed by phages*								No. of strains not lysed by
Azotobacter strains	tested	A-11	A-12	A-13	A-14	A-21	A-22	A-31	A-41	any phage
A. vinelandii	12	9	7	9	9	5	5	5	5	1
A. chroococcum	25	5	8	10	6	11	11	9	11	11
A. agilis	6	0	0	0	0	0	0	0	0	6
A. macrocytogenes	3	0	0	0	0	0	0	0	0	3
A. insigne	2	0	0	0	0	0	0	0	0	2
No. of strains tested	48	.	.	.	.	.	.	.	.	.
No. of strains lysed	.	14	15	19	15	16	16	14	16	.

* Only lytic reactions where plaque formation was observed are included.

DISCUSSION

The methods described for the isolation of the azotobacter phages are of special interest since they utilized the natural azotobacter flora for the enrichment strain rather than the usual addition of bacterial cultures. Although the majority of the soil samples were from various parts of Texas, phage isolates were made from Ohio and Maryland soils, indicating that the azotobacter phages are not confined to any particular geographical area of the United States. The fact that the phages from the Ohio and Maryland soils were antigenically related to phages isolated previously from Texas soils may indicate that the number of serological groups is not great. The four serological groups found thus far for the azotobacter phages are comparable to the number of groups reported for other phage systems.

The plaque morphology observed with the azotobacter phages differed markedly from that seen with the coli phages and many other phage systems; however, it resembled most closely the plaques formed by phage Kp which lyses *Klebsiella pneumoniae* type 2 (Park, 1956). The plaques formed by phage Kp were surrounded by translucent halos which gradually increased in diameter over the course of a week. It was demonstrated that a diffusible enzyme was liberated during lysis of the infected host cell, and that the enzyme was responsible for the large spreading halo that surrounded the phage plaques (Park, 1956; Adams & Park, 1956). It was believed that the enzyme decreased the viscosity of the capsular polysaccharide and stripped it from the surface of the bacterium, thus permitting the phage to penetrate the host cell surface.

The azotobacter phages produce plaques with halos, and since the *Azotobacter vinelandii* O organisms are capsulated, it is possible that a similar enzyme is present in the azotobacter + phage system and might account for the observed plaque morphology.

The most widely applied of the taxonomic criteria recommended for defining the species category in bacteriophages is serological relationship (Adams, 1959). Therefore primary emphasis was placed on serology in classifying the azotobacter phages. Cross-neutralization assays indicated four serological groups for the 10 azotobacter phages in the present collection. These groups were designated I, II, III and IV. The individual phages were given numbers preceded by the letter A which stands for the genus *Azotobacter*. We propose that the phages within a serological group be assigned numbers in the following manner: 11–19 for group I, 21–29 for group II, 31–39 for group III, and 41–49 for group IV. The purpose of using this range of numbers is to permit the incorporation of future phage isolates into any one of the present serological groups. In addition, it allows for inclusion of new serological groups (V, VI, etc.).

The kinetic studies on neutralization of phage with homologous and heterologous antisera indicate that phages A-21 and 23 in serological group II are identical or very closely related. These two phages are similar by plaque morphology and showed identical host-range specificities. If future investigations on additional classification criteria substantiate these findings, it is recommended that one of these two phages be dropped from the present series.

With phages A-12 and A-31 neutralization by homologous antisera did not follow first-order kinetics; however, such anomalous neutralization behaviour has not been uncommon with other phage systems. Andrews & Elford (1933) noted that neutralization of their phages proceeded rapidly until 90–99 % of the phage was inactivated and then slowed rather abruptly. They demonstrated that this phenomenon was not due to exhaustion of antibody since upon removal of phage the remaining serum was still capable of neutralizing phage. They subcultured plaques of phage particles which survived the neutralization experiment and showed that these were susceptible to antiphage serum neutralization and also exhibited similar anomalous neutralization behaviour. Delbrück (1945) observed a similar phenomenon with the T1 coli phage and suggested that antibody might be attaching itself at sites on the phage where it would not interfere with infectivity. Hershey & Bronfenbrenner (1952) believed that these anomalies might be due to an uncontrolled behaviour of complement. Thus, it is not clear whether the neutralization anomalies are a result of inherent heterogeneity of phage preparations or to the properties of specific lots of sera.

It was of interest to consider the value of the present series of phages in defining the bacterial species of the genus *Azotobacter*. Considerable disagreement exists as to whether the group of organisms designated *Azotobacter agilis* (*Bergey's Manual*, 1957) comprise one or two species, namely *A. agilis* and *A. vinelandii*. Some investigations have shown morphological, ecological and physiological differences between these two groups of organisms. The present studies indicate a difference between these strains since 11 out of 12 *A. vinelandii* strains showed lysis by one or more of the phages, whereas none of the six strains of *A. agilis* tested were lysed by any of the phages. Also, the strains designated *A. macrocytogenes* and *A. insigne*, which in many respects are similar to *A. agilis* cultures, were not lysed by any of the present phages. Since the *A. agilis* cultures have a distinct aquatic distribution and have never been isolated with certainty from the soil, it is possible that phages for these strains do not exist, or are rarely present, in the soil. Also, in the described phage isolation procedures the indicator strain used was a culture of *A. vinelandii*, a strain usually isolated from the soil. An exhaustive search for phages which lyse *A. agilis* strains using this culture as an indicator strain has not been made. The azotobacter phages described here are not species-specific since 14 out of 25 strains of *A. chroococcum* tested were lysed by one or more of the phages. Jones (1959) showed that at least one strain of *A. chroococcum* may be lysogenic. Although a considerable amount of discussion regarding taxonomy of the genus *Azotobacter* has centred around the relationship of *A. agilis* and *A. vinelandii*, two classification proposals (Tchan, 1953; Jensen, 1955) were concerned with the relationship of *A. chroococcum* and *A. vinelandii*. On the basis that these two species possessed a dormant stage (microcysts) they were included in the genus *Azotobacter* separate from *A. agilis*, which was placed in the genus *Azotococcus*. The present studies indicate a similar strain relationship, since the phages lysed *A. vinelandii* and *A. chroococcum*, but did not lyse strains of *A. agilis*.

We wish to express our appreciation to Mr Maxwell Nimeck for technical assistance and to Mr Phillip Hodson for taking photographs of the phage plaques. This work forms part of a thesis presented (by J.T.D.) for the Ph.D. degree to the University of Texas.

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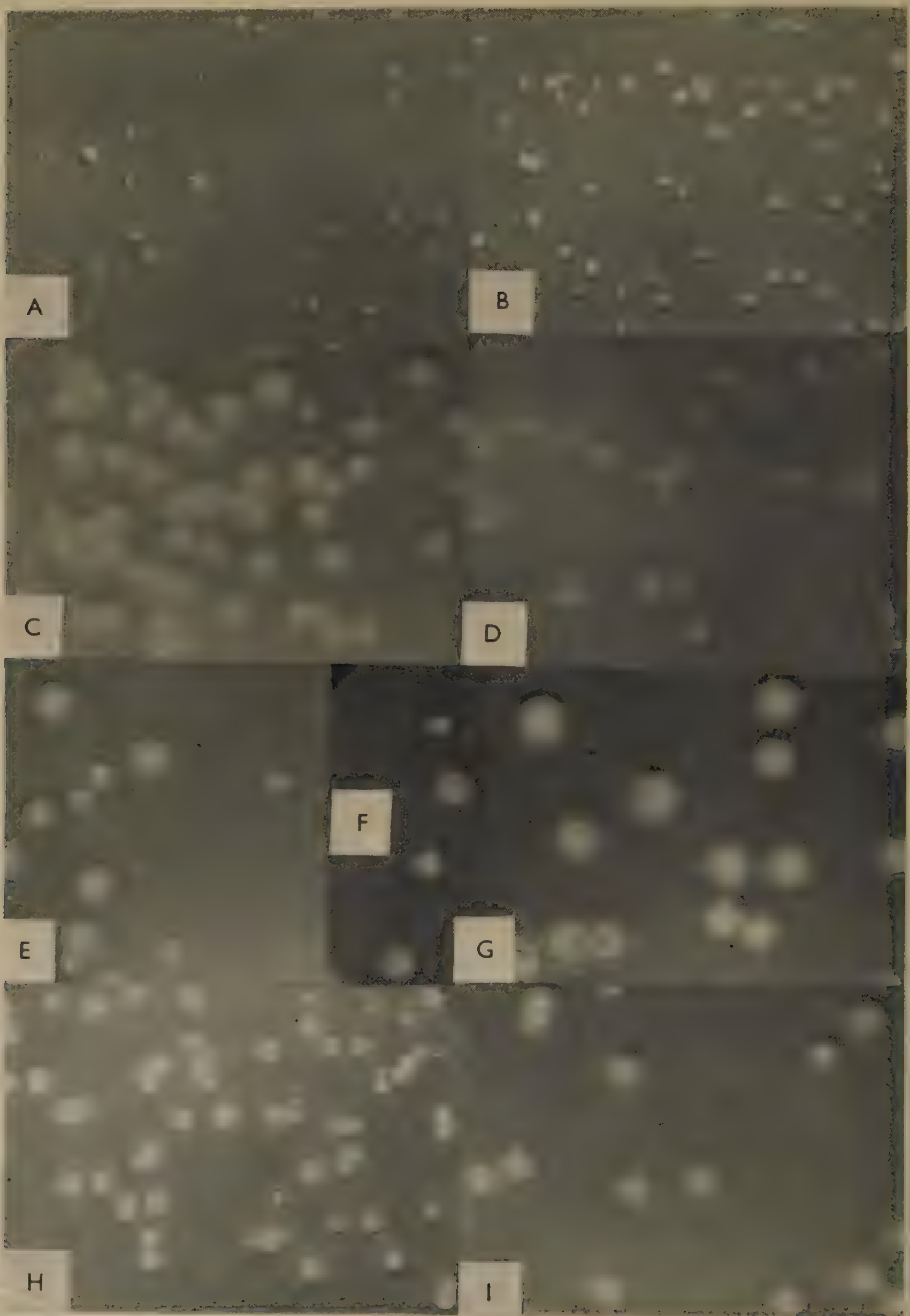

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EXPLANATION OF PLATE

Representative plaque types of azotobacter phages on host strain *Azotobacter vinelandii* O. Fig. A, phage A-12; fig. B, phage A-14; fig. C, phage A-13; fig. D, phage A-11; fig. E, phage A-22; fig. F, phage A-21; fig. G, phage A-23; fig. H, phage A-41; fig. I, phage A-31. Magnification *c.* ×1·5.

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On Sporulation in Sulphate-reducing Bacteria

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(*Received* 29 *August* 1960)

SUMMARY

Enrichment cultures of mesophilic sulphate-reducing bacteria were readily obtained from soils, mud and corrosion products which had been heated to 90°, yet pure cultures of *Desulfovibrio desulfuricans* were killed by heating to 60° even when absorbed on dried sterilized soil. Examination of the populations from heated samples revealed exclusively organisms resembling *Desulfovibrio orientis*; unheated samples contained either *D. orientis* alone or both *D. orientis* and *D. desulfuricans*. The belief that *D. desulfuricans* sporulates is questioned.

INTRODUCTION

Starkey (1938) observed that, though laboratory cultures of mesophilic sulphate-reducing bacteria were killed by heat, such organisms could nevertheless be obtained from soils which had been heated to 90° for ten minutes. Since he was able to demonstrate sporulation among the thermophilic sulphate-reducing bacteria, and since he supported the now obsolete belief that the mesophiles were adapted variants of the thermophiles (see Postgate, 1959*a*, for references), Starkey concluded that the mesophilic species *desulfuricans* formed spores in natural conditions but lost this ability after passage in laboratory media. Pochon & Chalvignac (1952) described a strain which showed such a change on microscopic observation. Starkey proposed the use of the generic name *Sporovibrio* on this account, but the oblique nature of the evidence for sporulation inhibited wide acceptance of this designation, *Desulfovibrio* being generally preferred. Prévot (1948), however, retained the name *Sporovibrio* and, in the third edition of his manual (Prévot, 1957), gave reasons for continuing to retain this generic name and for not accepting the exclusion of the sporulating thermophiles. A mesophilic species showing unequivocal sporulation was isolated by ourselves (Adams & Postgate, 1959) and named *Desulfovibrio orientis*. This species is readily differentiated from *D. desulfuricans* by two additional characters: (i) one or two polar or near-polar flagella (Adams & Postgate, 1959); (ii) absence of a fluorescence reaction typical of *D. desulfuricans* (Postgate, 1959*b*); it is distinguished from the thermophile *Clostridium nigrificans* by its mesophily and by the shape of its spores. It is possible that the heat resistance and sporulation earlier attributed to *D. desulfuricans* was due to the unrecognized presence of *D. orientis*; the present report provides evidence in favour of this view.

METHODS

Organisms and cultivation. Desulfovibrio desulfuricans, strain Hildenborough National Collection of Industrial Bacteria (NCIB) 8303, Essex 6 (NCIB 8307) and Beckton (NCIB 8319) were of fresh water origin; strains Canet 41 (NCIB 8393) and El Agheila Z (NCIB 8380) originated in saline environments and their culture media were supplemented with 2·5 % (w/v) NaCl. Stocks were kept in a freeze-dried condition; cultures for routine use were subcultured weekly in medium C of Butlin, Adams & Thomas (1949*b*) at 30° under H_2 99 % (v/v) + CO_2 1 % (v/v). *D. orientis*, strain Singapore (NCIB 8382), was maintained in a similar manner, but sodium thioglycollate (0·01 %, w/v) was added to its culture media.

Enrichment cultures of sulphate-reducing bacteria from natural samples were prepared in medium C supplemented with 0·01 % (w/v) sodium thioglycollate and 0·05 % (w/v) ferrous ammonium sulphate, pH 7·4 ± 0·3, in 1 oz. (*c*. 25 ml.) stoppered bottles filled to overflowing to exclude air. The thioglycollate ensured that *Desulfovibrio orientis* would grow if present (Adams & Postgate, 1959) and it also facilitated growth of *D. desulfuricans*, being below the concentration which showed inhibitory effects (Grossman & Postgate, 1953).

Diagnostic tests. Enrichment cultures were decanted to give bacterial suspensions reasonably free from FeS and the fluorescence test for *Desulfovibrio desulfuricans* (Postgate, 1959*b*) was performed on the decanted material. Flagella were observed microscopically after Casares-Gil staining. Thermophiles were sought by incubation at 55°. Motility was observed in wet preparations under phase contrast. Heat resistance was tested by sealing 1 ml. culture or 1 g. soil in a Durham tube and wholly immersing this in water at 90° for 10 min. Sterility of treated soils was tested by incubating portions aerobically and anaerobically in the glucose + peptone + yeast extract + salts medium of Postgate (1953).

Pure cultures were not in general isolated from the enrichment cultures. After 3–6 days at 30°, when the enrichment cultures were well blackened and rich in H_2S, the populations were assigned to *Desulfovibrio desulfuricans* or *D. orientis* groups according to the following criteria: *D. desulfuricans*, positive fluorescence test, vibrio form, single flagellum, translational motility, failure of subculture to grow at 55°; *D. orientis*, negative fluorescence test, fat vibrio form, more than one flagellum, motility absent or oscillatory, failure of subculture to grow at 55°.

RESULTS

We have repeatedly confirmed Starkey's observation that soils may be heated to 90° and still yield enrichment cultures of sulphate-reducing bacteria. On the other hand, the five pure strains of *Desulfovibrio desulfuricans* mentioned above were all killed when their cultures were heated to 60° or above for 10 min.; *D. orientis* usually survived heating to 90°. To see whether soil exerted a protective effect on *D. desulfuricans*, samples which had yielded sulphate-reducing bacteria after heating were sterilized (by autoclaving or by exposure to ethylene oxide at 4° for 30 min.), dried and then damped with cultures of the Hildenborough strain. The organisms were nevertheless killed when the damped soil was heated to 60° for 10 min. Since commensal spore-formers might have induced heat resistance in the manner that variants of *Clostridium sporogenes* enhance the heat resistance of *Clostridium welchii* (*Welchia perfringens*; Prévot, Raynaud & Tataki, 1951), three colonial types of sporulating anaerobe were isolated from heated enrichment cultures and mixed with the Hildenborough strain of *D. desulfuricans*. None conferred resistance to 10 min. at 60° upon it. Desiccation conferred a slightly increased heat resistance: a freeze-dried ampoule of the Hildenborough strain as supplied by the NCIB survived heating to 70° for 10 min., though others were killed at 80° and 90°.

It seemed likely that the heat-resistant types of sulphate-reducing bacteria were not of the species *Desulfovibrio desulfuricans*. Nineteen specimens of natural materials likely to contain these bacteria were collected and enrichment cultures made from them before and after heating for 10 min at 90°. All yielded flourishing cultures of sulphate-reducing bacteria. These were then assigned to the *D. desulfuricans* or *D. orientis* groups according to the criteria mentioned above. Although the cultures were not pure, and non-sulphate reducing bacteria were present, all the enrichments contained organisms which fell unequivocally into one or the other groups; intermediate types were not found.

Seven samples yielded cultures of the *Desulfovibrio desulfuricans* group when enriched without heating, but after 10 min at 90° they yielded only the *D. orientis* group. The samples tested comprised: corrosion products from a cooling system and a tubercle within a water pipe (cf. Butlin, Adams & Thomas, 1949*a*); a sewage sample from the Northern Outfalls Sewage Works, Beckton, London; scrapings from a mild steel plate held 1 ft. deep in the Thames at central London; mud samples from the Thames at Teddington and from Byron's pool at Cambridge; a soil sample from Pontardulais, Wales. Twelve specimens yielded the *Desulfovibrio orientis* group both before and after heating. All these were soil samples originating from: the laboratory grounds (2); a local compost heap; the Roman site at Catterick, Yorkshire (3); Singapore; Buenos Aires; Tanganyika; Hawaii; India (2). No soils of marine or brackish origin were tested. None of the enrichments grew on subculture at 55°.

DISCUSSION

These observations suggest that the heat-resistant sulphate-reducing bacteria in soils resemble *Desulfovibrio orientis* rather than *D. desulfuricans*. Consequently, the belief that the latter species sporulates in nature is probably mistaken. The relevance of these considerations to the inclusion of the species *desulfuricans* in a genus named *Sporovibrio* is evident.

We should like to draw attention to the fact that the *Desulfovibrio orientis* group was present in all specimens tested; in a majority of them it was the exclusive type of sulphate-reducing organism. This observation has little statistical value since the number of soils tested was small and their origins were not randomly distributed; nevertheless, it shows an unexpectedly high incidence. We attribute this to the use of thioglycollate in the enrichment media, without which *D. orientis* often fails to grow, whereas growth of *D. desulfuricans* is merely delayed. It seems likely that the *D. orientis* organism is more ubiquitous than was believed at the time of its isolation.

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The Occurrence and Location of Teichoic Acids in Lactobacilli

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SUMMARY

The walls and cell contents of a number of lactobacilli were prepared by mechanical disruption of the organisms followed by differential centrifugation. The nature of the teichoic acid present in the separated fractions was determined by extracting it with dilute trichloroacetic acid, precipitating with ethanol and identifying the precipitated polymer by hydrolysis to ribitol or glycerol phosphates and other recognizable degradation products. All the organisms contained a glycerol teichoic acid within the cell. This and similar observations with other bacteria indicates that glycerol teichoic acids play an important part in cellular metabolism. Both glycerol and ribitol teichoic acids occur in walls. The presence and type of teichoic acid in the walls correlate with the serological behaviour of lactobacilli and provide a useful means of classification. It is suggested that teichoic acids themselves may possess antigenic properties.

INTRODUCTION

The teichoic acids were first obtained from bacteria by extraction from whole defatted organisms with cold dilute trichloroacetic acid, followed by precipitation with ethanol (Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958). These preparations were mixtures containing the two types of this group of compounds: the glycerol teichoic acids are polymers of glycerophosphate bearing D-alanine residues in ester linkage with hydroxyl groups in the polymer, whereas the ribitol teichoic acids are similar polymers of ribitol phosphate bearing both D-alanine residues and sugars. The number and nature of the sugar residues and the configuration of the glycosidic linkages varies in the ribitol compounds from different organisms. Most of the structural details of the ribitol teichoic acid from *Bacillus subtilis* have now been determined (Armstrong, Baddiley & Buchanan, 1960).

Purified walls of *Bacillus subtilis*, *Lactobacillus arabinosus* 17-5, and *Staphylococcus aureus* H contain large amounts (40–60 % of their dry weight) of the appropriate ribitol teichoic acid but no glycerol teichoic acid, and it was concluded that the glycerol derivatives in material from whole organisms came from the cell contents. A more detailed survey of the presence of both types and their location in Lactobacillus was undertaken in an effort to understand their function and possible relationship to biochemical or serological properties of bacteria.

METHODS

Organisms. Most of the organisms studied were obtained originally from the National Collection of Industrial Bacteria (NCIB) and their identity was checked before use by physiological and biochemical tests. According to Sharpe (1957) *Lactobacillus plantarum* NCIB 3254 is *L. casei* in serological group B and *L. bulgaricus* NCIB 76 is *L. casei* var. *rhamnosus* serological group C. *L. delbrueckii* NCIB 8608 has not been classified serologically but it is a strain of *L. casei* var. *rhamnosus* very closely related to the NCIB 76 strain, and almost certainly belongs to serological group C.

Conditions for growth. Organisms were grown in batch culture (9 or 15 l.) in a liquid medium of the following composition: tryptone (Oxoid) 20 g.; yeast extract (Oxoid) 10 g.; sodium acetate 10 g.; glucose 20 g.; KH_2PO_4 4·5 g.; NaOH 1·04 g.; inorganic salts B (Barton-Wright, 1946) 5 ml.; oleic acid 0·01 ml.; Tween 40 1 ml.; L-histidine hydrochloride 100 mg.; distilled water 1000 ml.

Oleic acid was added for *Lactobacillus bulgaricus*, *L. delbrueckii*, *L. helveticus* and *L. lactis*, and L-histidine hydrochloride was added for rapid growth of *L. delbrueckii* (Ikawa & Snell, 1960). *L. bulgaricus*, *L. casei* var. *rhamnosus* (3 strains), *L. delbrueckii*, *L. fermenti*, *L. helveticus* and *L. lactis* were grown at 37° for 16 hr.; *L. plantarum* and *L. casei* at 30° for 16 hr.; *L. brevis* and *L. buchneri* at 30° for 45–48 hr.

Preparation of cell walls. Organisms were harvested in a refrigerated Sharples centrifuge, washed with cold physiological saline and suspended in cold distilled water (about 20 g. wet weight organism/100 ml. water). Disruption was carried out with a Mickle vibrator (Salton & Horne, 1951) and walls were collected by centrifugation. After washing and centrifuging several times with M-phosphate buffer (pH 7) and distilled water to remove traces of cytoplasmic material and whole organisms, the wall suspension was freeze-dried. The purity of the preparations was confirmed by electron microscopy.

Isolation of teichoic acid

From cell walls. Wall preparations were stirred with 10 % (w/v) trichloroacetic acid solution (1·5 ml. to 50 mg. cell wall) for 16 hr. at 4°. Walls were removed by centrifugation and extracted again with trichloroacetic acid solution for 24 hr. at 4°. Cold ethanol (2 vol.) was added to the combined extracts which were then kept at 4° for 24 hr. Precipitated teichoic acid was removed by centrifugation and reprecipitated from 10 % (w/v) trichloroacetic acid solution (about 5 ml.) by the addition of cold ethanol (1 vol.). After being kept at 4° for 24 hr. the precipitate was collected in a centrifuge, washed with cold acetone, ethanol and ether, then dried in a desiccator (vacuum). More prolonged extraction of walls at 4° yielded further small quantities of teichoic acid.

From cell contents. To the solution obtained after disruption of organisms and removal of walls by centrifuging was added cold 50 % (w/v) trichloroacetic acid solution to give a final concentration of 5 % (w/v) trichloroacetic acid. After 16 hr. at 4° the precipitate was removed by centrifuging and the clear solution was mixed with cold ethanol (2 vol.). The teichoic acid precipitated slowly at 4°; after 2–4 days it was collected and purified by the reprecipitation procedure described above for cell walls.

Identification. Samples of teichoic acid from walls or cell contents were hydrolysed with 2 N-hydrochloric acid for 3 hr. in sealed tubes at 100°. Hydrolysis products were identified by paper chromatography. Whatman no. 4 paper which had been washed in 2 N-acetic acid, then water, was used with the solvent systems *n*-propanol + ammonia (sp.gr. 0·88) + water (6 + 3 + 1) and *n*-butanol + ethanol + water + ammonia (sp.gr. 0·88; organic layer) (40 + 10 + 49 + 1). Products were detected by the spray reagents used previously (Armstrong *et al.* 1958). All the appropriate hydrolysis products of glycerol or ribitol teichoic acids were detected and no other products were seen. The presence of alanine ester linkages was confirmed in all samples by treatment with ammonia, followed by paper chromatography of the resulting alanine amide in the propanol solvent system (Armstrong *et al.* 1958).

RESULTS

The distribution of the two types of teichoic acid in the walls and cell contents of a number of *Lactobacillus* spp. is given in Table 1. The results are expressed qualitatively, and in each case refer to teichoic acid which had been isolated by the standard procedure and identified by chromatographic examination of hydrolysis products.

Table 1. *Teichoic acid in walls and cell contents of* Lactobacillus *spp. in relation to serological groups*

R = ribitol teichoic acid; G = glycerol teichoic acid; – = neither R nor G detected.

Organism	Teichoic acid in Cells	Teichoic acid in Walls	Serological group
L. arabinosus 17-5	G	R	D
L. plantarum NCIB 7220	G	R	D
L. brevis NCIB 8169	G	G	E
L. buchneri NCIB 8007	G	G	E
L. lactis NCIB 7278	G	G	E
L. bulgaricus NCIB 2889	G	G	A
L. helveticus NCIB 8025	G	G	A
L. plantarum NCIB 3254*	G	–	B
L. casei ATCC 7469	G	–	C
L. bulgaricus NCIB 76*	G	–	C
L. delbrueckii NCIB 8608*	G	–	C?
L. fermenti NCIB 6991	G	–	F
L. delbrueckii NCIB 8130	G	G	–

* A fuller description of these organisms is given under Methods.

DISCUSSION

All the organisms examined in this survey contained a glycerol teichoic acid in the fraction corresponding to the cell protoplasm and structure beneath the wall. It is concluded that glycerol teichoic acids probably occur in the cells of all *Lactobacillus* spp.; moreover, this type has been found in the cell contents of a variety of Gram-positive bacteria studied in this laboratory (Armstrong *et al.* 1959). In only two or three cases was this material not detected, and it is possible that these failures occurred through the insensitivity of the analytical procedure, which depends upon actual isolation of precipitated material. Alternatively, the occasional occurrence of fat-soluble teichoic acids containing higher fatty ester residues cannot be discounted; such compounds would escape detection by the methods adopted in this work.

Although the function of teichoic acids is unknown, the widespread occurrence of the glycerol type in Gram-positive bacteria establishes their importance, and the chemical reactivity of their alanine ester residues suggests a metabolic function for these polymers. It has not yet been possible to determine their exact location within the cell, but if they are present in the protoplast membrane or other outer regions of the cell they may be visualized as partners to the wall teichoic acids, the whole system being well suited to such functions as the transport of ions.

Both ribitol and glycerol teichoic acids are found in walls, but in some cases neither type was detected even when large samples of wall were examined. This finding, and the absence of intracellular glycerol teichoic acid in those walls which contained the ribitol compound, indicated that little or no cross-contamination of wall and cell material occurred during the isolation procedure. Faint traces of ribitol derivatives were detected in the cell contents of those organisms which possessed ribitol teichoic acid in their walls. It is not known whether this arose through contamination. Exhaustive chemical studies have not yet been carried out on all samples of teichoic acid obtained from Lactobacillus, but it is possible that structural differences occur within the two general types found in these organisms.

Some correlation was observed between the nature of the teichoic acid in the walls and the metabolic properties of the organisms. It is now generally recognized that, although the classification of *Lactobacillus* spp. solely upon metabolic behaviour is unsatisfactory, they may be classified well by consideration of both metabolic and serological behaviour (Sharpe, 1955; Sharpe & Wheater, 1957). Consequently, the serological groups of organisms studied in this work were compared with the teichoic acid of their walls. The serological groups listed in Table 1 are those described by Sharpe (1955) and Sharpe & Wheater (1957). It is seen that the organisms of group D contained ribitol teichoic acid in their walls, whereas the walls of those in groups E and A contained a glycerol teichoic acid; groups B, C, and F contained no wall teichoic acid. These results suggest a relationship between the serological properties of *Lactobacillus* spp. and the teichoic acid in their walls. It is likely then that these substances themselves would participate in serological reactions. Authentic samples of teichoic acids have not been examined serologically, but a closely related bacterial glycerophosphate polymer was studied by McCarty (1959) who was able to prepare a specific antiserum to it. Clearly, the serological behaviour of *Lactobacillus* spp. would not be determined solely by the antigenic properties of their wall teichoic acids. Thus, groups B, C and F are serologically distinct groups, but their members contain no wall teichoic acids. Nevertheless, a knowledge of wall teichoic acids should be valuable in classifying Lactobacilli, and might be used occasionally to predict the serological grouping of new members.

Ikawa & Snell (1960) detected teichoic acids in the walls of a number of *Lactobacillus* spp. Where identical strains have been used their results agree with those described here. However, they recorded the presence of a ribitol teichoic acid in the walls of *L. delbrueckii* 730 (ACTC 9649) which is understood to be identical with the strain NCIB 8130 (Sharpe, 1959). We found a glycerol teichoic acid in walls of

NCIB 8130 but no trace of a ribitol teichoic acid was detected. This small discrepancy is under investigation. Sharpe was unable to prepare an antiserum to this species but our observations would indicate its similarity to those of groups A or E.

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A Mutational Alteration of the Tryptophan Synthetase of *Escherichia coli*

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(*Received* 4 *October* 1960)

SUMMARY

A tryptophan auxotroph of *Escherichia coli* produced an altered tryptophan synthetase which could not convert indole to tryptophan but converted indole-3-glycerol phosphate to indole. As distinct from the normal tryptophan synthetase, which also catalysed this reaction, both pyridoxal phosphate and serine stimulated the activity of the mutant enzyme system. Fractionation and chromatography of the mutant tryptophan synthetase separated it into its two protein components, A and B. Examinations of the separated components showed that the A protein was normal, while the B protein was altered. Studies of the effect of serine on the pH-activity response of mutant preparations in the indole-3-glycerol phosphate → indole reaction demonstrated that different pH-activity responses were obtained, respectively, in the presence and absence of serine. The curves obtained were characteristic of the serine-requiring and serine-non-requiring reactions, respectively, of normal tryptophan synthetase. The saturation curves of the mutant component B by normal component A, with and without serine added, suggest that one role of serine and pyridoxal phosphate in the stimulation of the indole-3-glycerol phosphate → indole reaction is to bind together the A and B proteins in a catalytically effective complex.

INTRODUCTION

Studies performed with washed suspensions of two strains of *Escherichia coli*, a mutant requiring tryptophan for growth (strain 7-4) and a double mutant requiring tryptophan and serine or glycine (strain T2-15), indicated that serine was involved in the synthesis of indole from glucose and ammonium ion (Gibson, Jones & Teltscher, 1956). Although it was also observed that serine was required for anthranilic acid formation, subsequent experiments with bacteria fully adapted to anthranilic acid synthesis showed that serine participated at some point between anthranilic acid and indole, in indole formation by the mutant strains (F. Gibson, unpublished observation). It was not obvious which reaction required serine since in *E. coli* neither the conversion of anthranilic acid to indole-3-glycerol phosphate nor the conversion of indole-3-glycerol phosphate to indole normally involved this amino acid. Recently, DeMoss & Bonner (1959) described a mutationally altered tryptophan synthetase of *Neurospora crassa* (from strain td_{71}) which required serine and pyridoxal phosphate for the conversion of indole-3-glycerol phosphate to indole. Serine and pyridoxal phosphate are not required by the wild type enzyme in this reaction. In view of this finding it was thought that the unexplained effect of serine on *Escherichia coli* mutant 7-4 might be due to a similar alteration of its tryptophan synthetase. This possibility was examined and an attempt made to localize the alteration to one of the two protein components of the bacterial tryptophan synthetase.

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METHODS

Organisms. Three strains of *Escherichia coli* were used in this study. They were the parent strain (518), a mutant strain which required tryptophan for growth (7-4), and a double mutant derived from strain 7-4 which required tryptophan and serine or glycine for growth. These organisms have been described previously (Gibson *et al.* 1956).

Preparation of cell extracts. Organisms were grown on the mineral salts citrate mixture described by Vogel & Bonner (1956) supplemented with 0·16% (w/v) glucose and 0·005% (w/v) acid-hydrolysed casein (neutralized before use). The glucose was added as a sterile solution after sterilization of the medium. For *Escherichia coli* 7-4, L-tryptophan (5 μg./ml.) was included in the medium. One litre quantities of medium were inoculated with a 6–8 hr. culture in nutrient broth to give an initial population of about 10^6 organisms/ml. The cultures were then shaken vigorously at 37° for 16 hr. After harvesting the bacteria in a Sharples supercentrifuge they were washed once with cold 0·9% (w/v) NaCl solution and resuspended in 0·1 M 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer (pH 7·8, to give a final concentration of about 4×10^{11} bacteria/ml. The suspension was then disrupted in a 10 Kc Raytheon sonic oscillator for 20 min. Cell debris was removed by centrifugation at 144,000 *g* in a Spinco Model L centrifuge for 30 min. The supernatant fluid (crude extract) was stored at −15°.

In addition to crude extracts, preparations obtained by precipitating tryptophan synthetase three times with ammonium sulphate were used (ASP enzyme). Solid ammonium sulphate was added to the crude extract to obtain 50% saturation, the addition being made at 0–5° with stirring, and the stirring continued for a further 10 min. after solution. The precipitate was separated by centrifugation at 4600 *g* for 10 min. and dissolved in an amount of tris buffer (0·1 M; pH 7·8) equal to the original volume of extract. This precipitation was repeated twice and the final precipitate taken up in tris buffer and stored at −15°.

Reagents. Reagents with the exception of those given below were obtained commercially and not further purified. Indole-3-glycerol phosphate was prepared by the method described by Yanofsky (1956) modified to the extent that it was formed by the indole + triosephosphate reaction (Yanofsky, 1959). The solution used contained 1·8 μmole indole-3-glycerol phosphate/ml.; 1-(o-carboxyphenylamino)-1-deoxyribulose-5-phosphate (anthranilic deoxyribulotide) was prepared as described by Smith & Yanofsky (1960).

Normal A and B proteins from *Escherichia coli* T-3 and *E. coli* T-8, respectively, were purified on diethylaminoethyl (DEAE) cellulose columns (Crawford & Yanofsky, 1958). Proteins A and B from *E. coli* mutant strain 7-4 will be referred to as A_{7-4} and B_{7-4}.

Methods for assaying enzymic activity. The details of the methods used for estimating the activity of proteins A and B of tryptophan synthetase in the three reactions which they catalyse were described previously (Crawford & Yanofsky, 1958; Yanofsky & Stadler, 1958; Yanofsky, 1955, 1956). The conversion of indole-3-glycerol phosphate to indole was carried out in a reaction mixture consisting of indole-3-glycerol phosphate and phosphate buffer (pH 7). DL-serine and pyridoxal phosphate were added where desired. Indole was measured by reaction with Ehrlich's reagent as described by Yanofsky (1955). The conversion indole-3-glycerol phosphate → tryptophan was carried out in a mixture of indole-3-glycerol phosphate, DL-serine, pyridoxal phosphate, NaCl and tris buffer (pH 7·8). The reaction was followed by measuring the disappearance of indole-3-glycerol phosphate. The remaining indole-3-glycerol phosphate was oxidized to indole-3-aldehyde by metaperiodate at pH 5 and the absorption of indole-3-aldehyde formed was measured at 290 mμ. The conversion indole → tryptophan was carried out in the same reaction mixture as that just described except that indole was substituted for indole-3-glycerol phosphate. The reaction was followed by measuring indole disappearance. All tests were carried out in a final volume of 1 ml. at 37°.

One unit of tryptophan synthetase activity is defined as that amount of enzyme which catalyses the disappearance of 0·1 μmole substrate or the formation of 0·1 μmole product in 20 min. at 37°.

Chromatography. Column chromatography was carried out on Reagent Grade 'Selectacel' diethylaminoethyl (DEAE) cellulose (Brown Company, Berlin, New Hampshire, U.S.A.). The preparation and use of such columns for the separation and purification of the components of tryptophan synthetase were described by Crawford & Yanofsky (1958). In the present experiments a column 2 × 90 cm. was used. The gradient elution system consisted of 1 l. of 0·01 M-phosphate buffer (pH 7·0) in the mixing bottle and 1 l. of 0·4 M-phosphate buffer (pH 8·0) in the inlet bottle. Fractions of 7·5 ml. were collected at 18 min. intervals and the buffer was changed to 0·5 M-phosphate (pH 8·0) at fraction 173. All the buffers contained 2×10^{-5} M-pyridoxal phosphate and 10^{-4} M-reduced glutathione.

The crude extract was prepared for the column as follows. Nucleic acids were precipitated by addition of 1·9 ml. M-$MnCl_2$ to 25 ml. extract. After stirring for 15 min. the mixture was centrifuged and the precipitate removed (15 min., 4600 *g*). Then 9·67 g. $(NH_4)_2SO_4$ was added to 26 ml. of the supernatant solution and the mixture stirred for 10 min. After centrifugation (15 min., 4600 *g*) the precipitate was taken up in dialysis buffer (0·02 M-phosphate) and dialysed for 3 hr. against 1 l. of the same buffer. The final volume was 11·3 ml. of which 11 ml. was chromatographed. All operations were carried out at 0–5°.

Protein estimations. Protein was estimated by using the Folin phenol reagent as described by Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

The site of the serine effect

Indole is now considered to be a breakdown product of indole-3-glycerol phosphate rather than, as previously thought, a normal intermediate in tryptophan synthesis (Yanofsky, 1960). A number of bacterial mutants have been isolated which accumulate indole but, except in the case of *Escherichia coli* strain 7-4, there has been no report of serine being required for indole production. If the effect of serine on the production of indole by *E. coli* strain 7-4 were due to the fact that, as with *Neurospora crassa* mutant td_{71} (De Moss & Bonner, 1959), it stimulated the conversion indole-3-glycerol phosphate → indole, restriction of the available serine should cause the accumulation of indole-3-glycerol phosphate. Restriction of available serine could be achieved in two ways, either by using the double mutant which also required serine or glycine for growth (*E. coli* strain T2-15) or, alternatively, by using cell-free extracts which could not form serine from the available substrates.

Experiments with whole organisms. From a nutrient agar slope (24 hr., 37°) of the double mutant *Escherichia coli* strain T2-15, three flasks of mineral salts + citrate + glucose medium (10 ml. in 50 ml. flasks), each containing a different concentration of serine, were inoculated to give an initial population of about 2×10^6 bacteria/ml. The flasks were incubated for 18 hr. at 37° with vigorous shaking and then sampled for indole and indole-3-glycerol phosphate determinations. Table 1 shows that as the concentration of serine was decreased 20-fold the ratio of indole-3-glycerol phosphate to indole increased some 100-fold, indicating that serine probably did play some part in the conversion of indole-3-glycerol phosphate to indole by this strain.

Table 1. *The accumulation products of* Escherichia coli *strain T*2-15 (*double mutant requiring tryptophan and serine or glycine*) *when grown with limiting amounts of serine*

Organisms grown on basal medium* for 18 hr. with 5 μg. L-tryptophan/ml. and DL-serine as indicated.

Final concentration of DL-serine in basal medium (M)	Product formed (μmole/ml.)		Ratio: InGP/indole
	Indole	InGP†	
2×10^{-2}	9·1	0·45	0·049
10^{-2}	9·6	1·0	0·104
10^{-3}	0·026	0·15	5·8

* Medium as described in methods for preparation of cell extracts with the casein hydrolysate omitted.

† The accumulated material was probably predominantly indole-3-glycerol but was considered equivalent to InGP for the purposes of the experiment.

Experiments with cell extracts. The results given above were confirmed with cell extracts of *Escherichia coli* mutant strain 7-4 which were allowed to metabolize anthranilic deoxyribulotide, the intermediate immediately preceding indole-3-glycerol phosphate (Smith & Yanofsky, 1960). Table 2 shows that indole formation from anthranilic deoxyribulotide was stimulated 25-fold by the addition of serine. Furthermore, the conversion indole-3-glycerol phosphate → indole was increased fivefold when serine was added.

Examination of the effect of serine on indole formation

Indole formation in the presence and absence of serine. Table 2 shows that some indole was formed in the absence of serine when the ASP enzyme was incubated with indole-3-glycerol phosphate. Since the enzyme had been precipitated 3 times with ammonium sulphate it seemed unlikely that this indole formation was due to traces of serine remaining from the crude extract. It appeared more likely that

Table 2. *Effect of serine on the formation of indole-3-glycerol phosphate and indole by a cell-free extract of* Escherichia coli *strain* 7-4

Reaction mixture A consisted of anthranilic deoxyribulotide (0·44 μmole) and phosphate buffer (pH 7·9; 50 μmoles); mixture B contained indole-3-glycerol phosphate (0·18 μmole), phosphate buffer (pH 7·0; 50 μmole) and *Escherichia coli* strain 7-4 ASP extract (0·02 ml.) in a final volume of 1·0 ml. with the additions shown. Incubation was at 37° for 20 min. The contents of the tubes were then assayed for indole and indole-3-glycerol phosphate (InGP; see Methods).

	Product (mμmole)	
	InGP	Indole
Addition to reaction mixture A		
None	84	2
DL-serine (30 μmole)	32	50
Addition to reaction mixture B		
None	—	19
DL-serine (30 μmole)	—	100

some serine-independent indole formation was taking place. The components and additions involved in this serine-independent reaction will be called the basal system. Those mutant preparations and additions required to form indole in the presence of serine will be called the serine system. Hydroxylamine, a potent inhibitor of those reactions of tryptophan synthetase which require pyridoxal phosphate, slightly stimulates as a rule the conversion indole-3-glycerol phosphate → indole (Yanofsky & Rachmeler, 1958). Table 3 shows that the basal system was not affected by hydroxylamine, whereas in the serine system, hydroxylamine acted as an inhibitor, apparently competing with serine for the available pyridoxal phosphate. Inhibition by low concentrations of hydroxylamine provided indirect evidence that pyridoxal phosphate might also be involved with serine in the stimulation of the conversion of indole-3-glycerol phosphate to indole by this altered tryptophan synthetase.

Table 3. *Effect of hydroxylamine on indole formation by the basal and serine systems*

The reaction mixture contained: indole-3-glycerol phosphate, 0·18 μmole; phosphate buffer (pH 7·0), 50 μmole and in Expt. 1, 0·01 ml. crude *Escherichia coli* strain 7-4 extract, or in Expt. 2, 0·02 ml. of ASP enzyme together with various amounts of DL-serine and hydroxylamine as shown. Incubation time, 20 min. Final volume 1 ml.

	Substance added (μmole)		Indole formed (mμmole)
Experiment	DL-Serine	Hydroxylamine	
1	None	None	5
	None	1	4
	60	None	88
	60	1	5
2	None	None	4
	100	None	80
	100	0·01	71
	100	0·1	19
	100	1	5
	10	None	62
	10	0·01	23
	10	0·1	5
	10	1	5

Effect of pH *value on basal and serine reactions.* The pH value of the incubation mixture over the range pH 7—9 (Fig. 1) affected the basal and serine systems differently. The curves obtained may be compared with those in Fig. 2 obtained with normal tryptophan synthetase. The curve for indole formation by the basal system (Fig. 1) resembles that for the conversion indole-3-glycerol phosphate → indole by normal tryptophan synthetase (Fig. 2). The curve for indole formation by the serine system (Fig. 1) more closely resembles that for the serine and pyridoxal

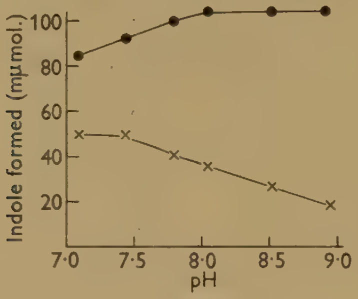

Fig. 1

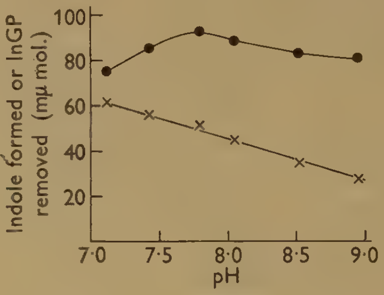

Fig. 2

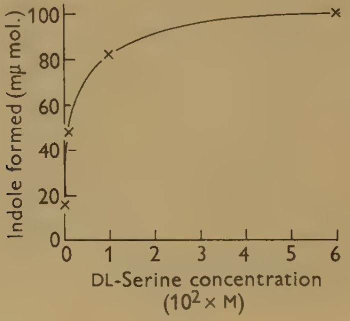

Fig. 3

Fig. 1. The effect of pH on the conversion indole-3-glycerol phosphate → indole by the serine and basal systems with *Escherichia coli* strain 7-4 extract. The reaction mixtures were: for serine system, InGP (0·18 μmole), DL-serine (60 μmole), pyridoxal phosphate (0·03 μmole), normal A (86 units*) crude *E. coli* 7-4 extract (0·01) ml.; for basal system, InGP (0·18 μmole), hydroxylamine (1 μmole), normal A (86 units*) crude *E. coli* strain 7-4 extract (0·04 ml.) together with 0·5 M-tris buffer (50 μmole) in final volume 1 ml. Incubation times: serine system 20 min.; basal system 30 min. Serine system, ●—●; basal system, ×—×.

* Units measured in indole → tryptophan reaction.

Fig. 2. The effect of pH value on the conversion indole-3-glycerol phosphate (InGP) → tryptophan and InGP → indole by normal tryptophan synthetase. The reaction mixtures were: for InGP → tryptophan reaction; InGP (0·18 μmole), DL-serine (60 μmole), pyridoxal phosphate (0·03 μmole), saturated aqueous NaCl solution (0·03 ml.), normal component A (10 units*), normal protein B (2 units*); for InGP → indole reaction; InGP (0·18 μmole), hydroxylamine (1 μmole), normal component A (150 units*), normal component B (40 units*), with tris buffer (50 μmole). Incubation time 20 min. Final volume 1 ml. InGP → tryptophan, ●—●; InGP → indole, ×—×.

* Units measured in indole → tryptophan reaction.

Fig. 3. The effect of serine concentration on the rate of indole formation. The reaction mixture contained indole-3-glycerol phosphate (0·18 μmole), phosphate buffer, pH 7·0 (50 μmole), ASP enzyme from *Escherichia coli* strain 7-4 (0·02 ml.) with serine as indicated. Final volume 1 ml.; incubation time 20 min.

phosphate-dependent formation of tryptophan by normal tryptophan synthetase (Fig. 2).

Specificity of L-*serine.* Some amino acids were tested as possible substitutes for DL-serine in the serine system (Table 4). L-Serine was twice as active as DL-serine on a molar basis, while D-serine had a slight stimulatory effect; this may have been due to contamination of the D-serine with small amounts of L-serine. The other amino acids tested were inactive.

Table 4. *Indole synthesis with various amino acids related to serine*

The reaction mixture was as in Table 3. Enzyme from *Escherichia coli* strain 7-4 (ASP preparation, 0·02 ml.) with the amino acids as shown.

Addition	Final concentration (M)	Indole formed (mμmole)
None	—	10
DL-Serine	0·06	93
L-Serine	0·03	100
D-Serine	0·03	25
DL-Homoserine	0·06	10
DL-Threonine	0·06	8
L-Cysteine	0·03	14
Glycine	0·03	14

Serine saturation curve. Figure 3 shows the effect of increasing concentrations of serine on indole formation from indole-3-glycerol phosphate. The K_A for DL-serine estimated from the curve is about $1{\cdot}3 \times 10^{-3}$ M which is of the same order as the K_M for serine with normal tryptophan synthetase (K_M for L-serine $= 1{\cdot}7 \times 10^{-3}$ M; C. Yanofsky, unpublished).

Pyridoxal phosphate and the serine reaction

The inhibition of the serine stimulation by hydroxylamine was strong presumptive evidence that pyridoxal phosphate was concerned in the serine system. However, indole formation by the ASP enzyme was only stimulated to a variable small degree or not at all by added pyridoxal phosphate, despite the fact that ammonium sulphate precipitations usually removed much of the bound pyridoxal phosphate (C. Yanofsky, personal communication). However, after dialysis of 0·5 ml. of ASP preparation for 2 hr. against 1 l. of 0·02 M tris buffer (pH 7·8) it was possible by adding pyridoxal phosphate in the serine system to show stimulation of indole formation (1·5-fold) by mutant preparations.

Separation of the A and B proteins of the tryptophan synthetase of Escherichia coli *mutant strains* 7-4

The two protein components A and B of *Escherichia coli* tryptophan synthetase can be separated by chromatography on cellulose ion exchangers (Crawford & Yanofsky, 1958). To learn more about the peculiarities of the tryptophan synthetase of *E. coli* strain mutant 7-4 the two component proteins were separated and examined individually. After chromatography of a cell-free extract of *Escherichia coli* strain 7-4 on DEAE cellulose (see Methods) the fractions obtained were assayed for component A activity in the indole → tryptophan reaction in the presence of an excess (10 units) of normal B protein (Fig. 4*a*). After the first peak of component A ($A_{7\text{-}4}$) was located the fractions were examined for component B by measuring the conversion indole-3-glycerol phosphate → indole (in the presence of serine and an excess of $A_{7\text{-}4}$). Figure 4*b* shows that $B_{7\text{-}4}$ activity was detected in two peaks. Examination of fraction 154 indicated that the addition of $A_{7\text{-}4}$ was not necessary for this fraction to catalyse the conversion indole-3-glycerol phosphate → indole. Therefore, the first $B_{7\text{-}4}$ peak probably represented an AB complex (Crawford & Yanofsky, 1958). This was confirmed by assaying fractions 140 to 190 for component A activity using excess normal B protein and measuring the conversion indole → tryptophan (Fig. 4*a*). The second peak of $B_{7\text{-}4}$ activity contained only low amounts of $A_{7\text{-}4}$. Both $A_{7\text{-}4}$ and $B_{7\text{-}4}$ were concentrated by pooling the appropriate fractions, precipitating the proteins with ammonium sulphate at 60% of saturation, and redissolving the precipitates in 0·1 M-tris buffer (pH 7·8). Purified $B_{7\text{-}4}$ protein was stored in buffer containing pyridoxal phosphate and reduced glutathione (see Methods).

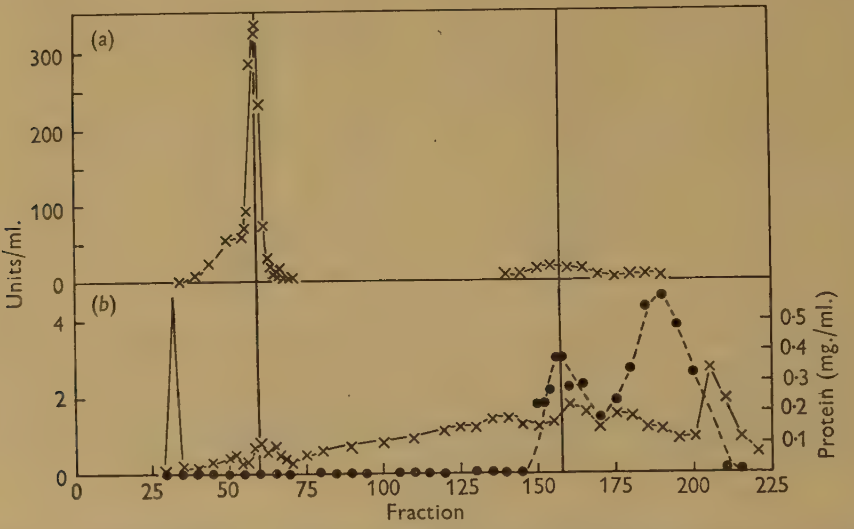

Fig. 4. Chromatography of tryptophan synthetase from *Escherichia coli* strain 7-4 on DEAE cellulose. Details of assay methods in text. (*a*) Component A activity, ×—×; (*b*) component B activity, ●—●; protein concentration, ×—×.

Experiments with the separated $A_{7\text{-}4}$ *and* $B_{7\text{-}4}$ *components*

Component $A_{7\text{-}4}$ appeared to be a normal A protein; in the presence of normal B protein it catalysed all reactions characteristic of normal A protein. Component $B_{7\text{-}4}$ was obviously an altered component; in the presence of $A_{7\text{-}4}$ or other normal component A it failed to convert indole to tryptophan. Furthermore, serine stimulated the conversion indole-3-glycerol phosphate → indole by a mixture of $B_{7\text{-}4}$ and normal A protein.

The addition of serine to a mixture of normal A and B proteins in the presence of indole-3-glycerol phosphate and pyridoxal phosphate eliminated indole formation from indole-3-glycerol phosphate because of the much faster conversion of the latter to tryptophan (Table 5).

On the other hand, a mixture of $B_{7\text{-}4}$ and normal A protein formed indole from indole-3-glycerol phosphate both in the presence and absence of serine (Table 5) as expected on the basis of the experiments with the ASP extract. Purified $B_{7\text{-}4}$ protein had some activity when measured in the serine system without added A protein (Table 5); this can be explained by the small amount of $A_{7\text{-}4}$ that was present (see Fig. 4).

Table 5. *The effect of serine on the conversion of indole-3-glycerol phosphate to indole with* $B_{7\text{-}4}$ *protein and the components of normal tryptophan synthetase*

The reaction mixture consisted of the reagents listed in the Table together with indole-3-glycerol phosphate (0·18 μmole) and phosphate buffer (pH 7·0; 50 μmole). In Expts. 1 and 3 (*a*), hydroxylamine (1 μmole) was added; in Expts. 2 and 3 (*b*), pyridoxal phosphate (0·03 μmole) was added. Final volume 1 ml. Incubation times: Expt. 1, 80 min.; Expt. 2, 30 min.; Expt. 3, 30 min.

Experiment	Normal A protein (units)*	A_{7-4} protein (units)*	Normal B protein (units)*	B_{7-4} protein (ml.)	Serine added (μmole)	Indole formed (mμmole/ 20 min.)
1 (*a*)	560	—	—	0·02	—	22
(*b*)	—	—	—	0·02	—	< 1
2 (*a*)	56	—	—	0·02	60	87
(*b*)	—	—	—	0·02	60	5
3 (*a*)	—	26	80	—	—	31
(*b*)	—	13	40	—	60	< 1

* Units measured in the indole → tryptophan reaction. In Expts. 1 (*a*) and 2 (*a*) the normal A protein added was sufficient to saturate the B_{7-4} protein used.

It was impossible to obtain reaction rates for the three reactions catalysed by tryptophan synthetase with either of the two components in excess, since the $B_{7\text{-}4}$ protein of course failed to catalyse the indole → tryptophan reaction. Further, this B protein could not be used in excess in the basal system, since at the concentration of enzyme required the preparation became inhibitory to the reaction.

The ratio of reaction rates for the indole → tryptophan and indole-3-glycerol phosphate → indole reactions with normal A protein and excess normal B protein in the first case, and normal A and excess $B_{7\text{-}4}$ plus serine and pyridoxal phosphate in the second case, was 1:0·05. The ratio of reaction rates for the same two reactions using normal A protein with normal B component was 1:0·03 (Yanofsky, 1960). It is clear then, that the addition of serine to the mutant system restored the ratio to nearly normal but did not accelerate the indole-3-glycerol phosphate → indole reaction to a point where it would be comparable with the serine requiring reactions carried out by normal A and B components.

Saturation of $B_{7\text{-}4}$ *by normal A.* With normal tryptophan synthetase components about a three-fold excess of one component was necessary to saturate fully the other component for maximal activity in the conversion indole → tryptophan (Crawford & Yanofsky, 1958). In Fig. 5 it can be seen that the curve for the saturation of normal protein B by normal A in the conversion indole-3-glycerol phosphate →

indole was almost identical with that obtained for the conversion indole → tryptophan (Fig. 5).

It was of interest to examine the saturation curves for B_{7-4} protein in both the serine and basal systems (Fig. 6). A striking difference was detected; in the absence of serine 25 times more protein A was required for half saturation of B_{7-4} than in its presence.

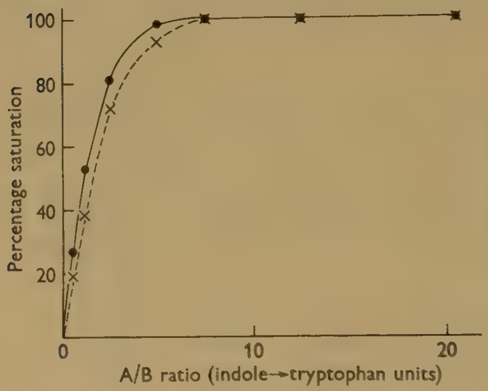

Fig. 5

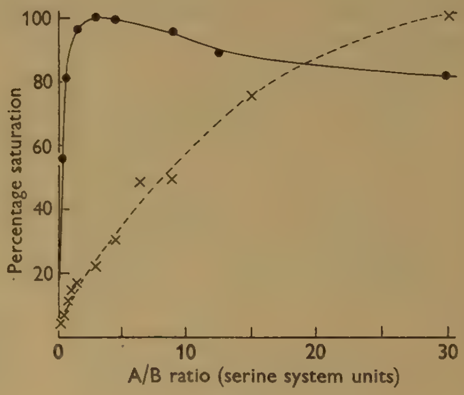

Fig. 6

Fig. 5. The saturation of normal B protein by normal A protein as measured in the conversion indole → tryptophan and the conversion indole-3-glycerol phosphate (InGP) → indole. The reaction mixtures were: (*a*) indole → tryptophan: phosphate buffer pH 7·0 (50 μmole), indole (0·4 μmole), DL-serine (60 μmole), pyridoxal phosphate (0·03 μmole), saturated aqueous NaCl solution (0·03 ml.), normal B (3·2 units), various amounts of normal A protein; (*b*) InGP → indole: InGP (0·18 μmole), phosphate buffer pH 7·0 (50 μmole), hydroxylamine (1 μmole), normal B (26 units), various concentrations of normal A protein. Incubation time 20 min. for both tests. Units are measured in the indole → tryptophan reaction. Indole → tryptophan, ●—●; InGP → indole, ×---×.

Fig. 6. The saturation of B_{7-4} protein by normal A protein in the serine and basal systems. The reaction mixtures were: for serine system; indole-3-glycerol phosphate (0·18 μmole), DL-serine (60 μmole), pyridoxal phosphate (0·03 μmole), phosphate buffer pH 7·0 (50 μmole), purified B_{7-4} protein preparation (0·03 ml.), and normal B protein as indicated; for basal system; as above with serine and pyridoxal phosphate omitted and hydroxylamine (1 μmole) added. Final volume 1 ml.; incubation times: serine system 30 min.; basal system 80 min. Serine system, ●—●; basal system, ×---×.

DISCUSSION

It seems probable from the findings reported here that the mutation which affects the tryptophan synthetase in *Escherichia coli* 7-4 leads to the production of an altered enzyme system with characteristics similar to those of the tryptophan synthetase of the mutant of *Neurospora crassa* described by DeMoss & Bonner (1959). Experiments with whole organisms and cell extracts of *E. coli* strain 7-4 have shown that serine and probably pyridoxal phosphate have a marked effect on the conversion indole-3-glycerol phosphate → indole although neither of these compounds has any effect on this reaction with normal tryptophan synthetase. The present observations thus extend the similarities of mutational effects on this enzyme system in the two organisms. A major difference between the two systems, however, is the ready dissociation of the tryptophan synthetase from *E. coli* into two protein components A and B (Crawford & Yanofsky, 1958). This property of the *E. coli* system has permitted the localization of the mutational defect in *E. coli* strain 7-4 to the B protein. The participation of serine and pyridoxal phosphate in a reaction involving an altered B protein lends further support to the suggestion of Crawford & Yanofsky (1958) that protein B provides the surface on which the serine + pyridoxal phosphate complex is combined during those reactions of tryptophan synthetase in which this complex is involved.

Three types of experiments differentiate the conversion indole-3-glycerol phosphate → indole in the presence and in the absence of serine: (i) the serine system is strongly inhibited by a low concentration of hydroxylamine; (ii) the pH-activity curves for the two systems are different; (iii) curves for the saturation of $B_{7\text{-}4}$ protein by normal A protein have marked differences in the serine and basal systems.

The function of serine (presumably as a Schiff base with pyridoxal phosphate) in the serine system is of interest. It does not act as a substrate in the conversion indole-3-glycerol phosphate → indole but it is possible that it is changed to some other compound by the A B protein complex during the course of the reaction. It was thought that if serine were attacked during indole formation, pyruvate would be a likely end product. Experiments designed to detect pyruvate production by the specific method of Friedmann & Haugen (1943) were clearly negative. The affinity for serine is fairly low (K_a about $1{\cdot}3 \times 10^{-3}\,\mathrm{M}$) and with the concentrations of serine necessary to obtain a significant reaction it was not practicable to look for serine removal,

It has been suggested (DeMoss & Bonner, 1959) that in mutants of *Neurospora crassa* similar to *Escherichia coli* strain 7-4, serine and pyridoxal phosphate might be required to maintain the active site of the altered enzyme in its 'proper' configuration for cleavage of indole-3-glycerol phosphate. This suggestion is supported and extended by the experiment shown in Fig. 4. The amount of normal A protein required for half saturation of $B_{7\text{-}4}$ protein in the basal system is about 25 times that required for the serine system. This suggests that one function of serine and pyridoxal phosphate in the conversion indole-3-glycerol phosphate → indole by the tryptophan synthetase of *E. coli* strain 7-4 is to assist in binding the A and B components into an enzymically effective complex.

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Virolysin, a Virus-Induced Lysin: Its Appearance and Function in Phage-Infected Staphylococci

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SUMMARY

The formation and role of enzyme, virolysin, in *Staphylococcus aureus* K_1 infected with phages P_1 and P_{14} are described. Virolysin is a by-product of the metabolism of the cell which is actively producing phage, not of the normal cell. Virolysin is first detected within 10–15 min. in a 40–50 min. latent period and increases linearly until lysis. Normal cell autolysin remains constant during infection. Observations on lysis and phage release show that (1) certain inhibitors which prevent lysis of the cocci by external virolysin also prevent lysis and phage release when added at the end of the latent period; (2) the rate of premature lysis of, and phage release from, cocci chilled during the latent period depends upon their virolysin content. Both observations suggest that virolysin functions in phage release.

INTRODUCTION

Lysins obtained from phage-infected bacteria were reported by Bronfenbrenner & Muckenfuss (1927) for a staphylococcal system, by Sertić (1929) for a coliphage system, and by Humphries (1948) for a klebsiella system. More recent publications describing phage-induced cell-wall or capsule-dissolving agents are those of Panijel & Huppert (1954) for an *Escherichia coli* system, Ralston and collaborators (Ralston, Baer & Krueger, 1955*a*; Ralston, Baer, Lieberman & Krueger, 1955*b*, 1957*a*; Ralston, Lieberman, Baer & Krueger, 1957*b*) for a *Staphylococcus aureus* system; and Adams & Park (1956) for klebsiella, Murphy (1957) for *Bacillus megaterium*, Maxted (1957) for streptococci, Koch & Dreyer (1958) for *E. coli* bacterium + bacteriophage systems. In addition to virus-induced lysins, other instances of virus-induced enzymes include DNA-synthesizing enzymes in *E. coli* B-T phage systems (Kornberg, Zimmerman, Kornberg & Josse, 1959; Flaks & Cohen, 1959; Flaks, Lichtenstein & Cohen, 1959), adenosine triphosphatase associated with avian myeloblastosis virus (Mommaerts *et al.* 1952) and the amidase of influenza virus (Gottschalk, 1952) in animal host + virus systems.

Virolysin is a lysin found in lysates of *Staphylococcus aureus* K_1 infected with phage P_1. In previous publications (Ralston *et al.*, 1955*a*, *b*; 1957*a*, *b*) we showed the following features: virolysin has the properties of an enzyme; it cannot be found in uninfected cocci but a similar enzyme, autolysin, is present. The two enzymes differ in their substrate specificity, antigenicity and pH optimum. For virolysin to act externally the cocci must be pretreated with phage, heat, acetone, urea or a variety of other damaging agents. We suggested that: (1) virolysin acts on a substrate forming the framework of the bacterial wall; (2) in order for the substrate to become available to the enzyme, a preliminary alteration (presumably of the wall) must occur.

Preliminary studies (Ralston *et al.*, 1955*b*) suggested that virolysin appeared intracellularly during phage infection and that it acted in the final lysis to release virus. The present work was undertaken to obtain further information about the relationship between phage infection, virolysin appearance and the role of virolysin in lysis. More specifically, it would be of interest to determine (1) when and how the enzyme appears in the infected coccus; (2) what effect phage infection has on the quantity of normal cell autolysin; (3) whether virolysin can be induced in the absence of phage or whether these syntheses are keyed one to the other; (4) whether phage release is dependent upon the presence and operation of the enzyme at the end of the latent period.

METHODS

Media. Tryptose phosphate broth or agar (Difco Labs., Detroit, Mich., U.S.A.) was used for growth of bacteria and studies on phage-infected cells. In this paper, the media are referred to as TP 1 × or 2 × broth (i.e. single or double strength broth) and TP agar.

Bacterium + bacteriophage systems and phage assay. The phages P_1 and P_{14} and their bacterial hosts *Staphylococcus aureus*, strains K_1 and 145, were described previously (Ralston & Krueger, 1952, 1954). The method of assaying phage by a rapid plaque technique on the surface of microscope slides was previously reported (Jones & Krueger, 1951; Ralston & Baer, 1960). The following abbreviations are used throughout: P = phage; C = cocci; $P_{14}(K_1)$ = phage 14 produced on strain K_1 cocci and $P_1(145)$ = phage 1 produced on strain 145 cocci, and so forth.

Preparation of lysins. To prepare autolysin, 1×10^8 resting uninfected K_1 C/ml. were shaken in TP 2 × broth for 6 hr. at 37° (final concentration = 3×10^9 C/ml.) and stored at 4° until autolysis occurred (24–72 hr.). Preparation of virolysin $P_1(K_1)$ involved shaking 1×10^8 resting K_1 C/ml. with 1×10^6 P_1 phage/ml. in TP 2 × broth at 37°. Lysis of the culture occurred in 3 hr. The autolysates and lysates were spun at 20,000*g* for 1 hr. at 6°. The K_1 autolysin and $P_1(K_1)$ virolysin were present in the supernatant fluids.

Determination of lysin activity. Virolysin and autolysin assays were performed by turbidimetric measurement of the lysis of heat-killed K_1 cocci under standardized conditions. The enzyme concentration in any particular sample of autolysin or virolysin was determined by mixing a suitable dilution of lysin with heat-killed K_1 cocci at 37° (sometimes, however, at 4°). Generally a 1/2 to 1/100 dilution of lysin in TP 2 × broth at pH 7·5 for virolysin, and pH 6·5 for autolysin, was mixed with cocci so that the final volume in the tube was 5 ml. and the final concentration of cocci was 1×10^9 C/ml. The decrease in turbidity was followed in a Klett-Summerson photoelectric colorimeter by taking readings at suitable intervals. From the data, the course of lysis can be represented by plotting the logarithm of the number of unlysed cocci against time at any given temperature. Only half of the heated cocci lysed and a correction was made for the resistant cocci in the population. A straight line was generally obtained (Ralston *et al.*, 1957*b*). Estimates of lysin activity were based upon determinations of the initial velocity constants, K/min., calculated from the equation:

$$K = \frac{2 \cdot 3}{t} \log \frac{C_0}{C_0 - C_t},$$

in which C_0 equals the initial concentration of cocci/ml., C_t equals cocci/ml. lysed in any given time interval, t. These relative velocity constants are reported only for purposes of comparison within an individual experiment. Less accurate estimates of lysin activity were made by determining the % cocci lysed in a short time interval, and comparing the % lysis instead of the velocity constants, K, of individual samples.

Preparation of virolysin antiserum. A phage $P_1(K_1)$ lysate was spun at 20,000*g*, for 1 hr. at 6° in a Spinco Model L centrifuge. The pellet containing the phage was discarded. Solid $(NH_4)_2SO_4$ was added to the supernatant fluid at 0° to 40 % of saturation. The precipitated material was centrifuged at 4000 rev./min. for 15 min. and resuspended in 0·85 % (w/v) NaCl. At least a tenfold concentration of the original activity was obtained. A rabbit was inoculated subcutaneously with progressively higher amounts (0·5–2·0 ml.) of the concentrated material in a series of sixteen injections over 3–4 weeks. Serum was collected 2 weeks after the last injection. A second rabbit was inoculated with autolysed cell contents, which exhibited no lysin action, to determine whether anything in the cell contents contained antigens similar to virolysin. A number of rabbit sera tested, including several phage antisera, contained a lysin for heated K_1 staphylococci. This lytic activity was not removed by heating the sera at 56° for several hours, but was radically diminished by filtering the sera through Supercel filter-aid.

Antiserum inhibition tests. Antiserum to autolysin or virolysin was used at a dilution of 1/25 and mixed with enzyme for 20 min. at 20° before adding heat-killed bacteria. The enzyme control contained enzyme mixed with a similar dilution of normal rabbit serum.

Specificity of virolysin antiserum. From Tables 1 and 2 it is seen that: (1) antiserum to either P_1 or P_{14} virolysins (produced on strain K_1) did not affect the normal K_1 cell autolysin, indicating that the virolysin was antigenically unrelated to autolysin; (2) the antibody to $P_1(K_1)$ virolysin inactivated virolysin formed in either strain K_1 or strain 145 cocci, indicating that the antigenic specificity of the virus-induced enzyme depended on the phage rather than the host of production; (3) antiserum to the virolysin induced by the closely related phage P_{14} also inactivated P_1 virolysin, regardless of the host, showing that the differences between the phages did not in-

Table 1. *Staphylococcal virolysin. Effect of antiserum to $P_1(K_1)$ virolysin on $P_1(K_1)$ virolysin and staphylococcus strain K_1 autolysin*

	K/min. at 37°
Virolysin + inactive autolysin	0·026
Autolysin + inactive virolysin	0·010
Autolysin + inactive virolysin + virolysin antiserum	0·008
Virolysin + autolysin	0·039
Virolysin + autolysin + virolysin antiserum	0·010

volve genes concerned with virolysin induction. K_1 autolysin antiserum is specific for autolysin and does not affect virolysin (Lieberman, 1956; Ralston *et al.*, 1957*b*). The following controls gave no lysis of heated cocci: inactive virolysin + inactive autolysin; these two + virolysin antiserum; virolysin + inactive autolysin + virolysin antiserum. Inactive lysin was obtained by boiling a lysin sample for 5 min.

Table 2. *Staphylococcal virolysin. Effect of antiserum to $P_{14}(K_1)$ virolysin on $P_{14}(K_1)$, $P_1(K_1)$, and $P_{14}(145)$ virolysins*

Test mixture	Virolysin from P_{14} infection of strain K_1	Virolysin from P_1 infection of strain K_1	Virolysin from P_{14} infection of strain 145
	K/min. at 37°		
Enzyme	0·0204	0·0530	0·0154
Enzyme + antiserum to $P_{14}(K_1)$ virolysin	0·0000	0·0000	0·0000

Analysis of total lysin, autolysin and virolysin content of phage-infected and uninfected cocci. Total lysin (virolysin + autolysin) in phage-infected cocci can be assayed on heated K_1 cocci. The relative velocity constants, *K*/min., of a mixture of the two has been shown to be equal to the sum of the activities of the individual lysins (Ralston *et al.*, 1957*b*). Autolysin in a mixture of the two lysins can be determined in three ways: (1) K autolysin $= K_{\text{total lysin}} - K_{\text{residual lysin}}$ in presence of autolysin antibody; or (2) $K_{\text{residual lysin}}$ in presence of virolysin antibody; (3) by assay on acetone-treated *Micrococcus lysodeikticus*, which is lysed by autolysin but not by virolysin. Virolysin is determined by: (*a*) K virolysin $= K_{\text{total lysin}} - K_{\text{residual lysin}}$ in presence of virolysin antibody; (*b*) $K_{\text{residual lysin}}$ in presence of autolysin antibody.

RESULTS

Virolysin production in strain K_1 staphylococci during multiplication of phage P_1

The following two experiments were designed to determine the appearance and increase of virolysin in the phage-infected cocci, to ascertain changes, if any, in the autolysin content of the cocci after infection, and to compare these with the appearance and increase of mature phage. The experiments differed from each other in the phage used and in the method used to obtain and assay the intracellular contents; the results of the two experiments corroborate each other.

Experiment 1. Staphylococci (strain K_1), grown for 4 hr. at 37°, were suspended at $2{\cdot}5 \times 10^8$ C/ml. in TP broth, and infected with P_1 phage at a phage:coccus ratio of 4:1. The phage + cocci mixture was allowed to remain at room temperature for 3 min. and then shaken at 37°. Samples were chilled at intervals and tyrothricin was added to 0·001 mg./ml., to cause lysis (Fong & Krueger, 1950); the concentration of tyrothricin used did not inactivate free phage or lysin. Lysin activities were determined by using virolysin antiserum and by assays on *Micrococcus lysodeikticus*.

Experiment 2. Staphylococci (strain K_1) were grown on TP agar at 37° for 4 hr., suspended in TP $2\times$ broth to $2{\cdot}5 \times 10^8$ C/ml., chilled to 4°, and infected with phage 14(K_1) at a phage:coccus ratio of 4:1. After 20 min. adsorption at 4°, the infected cocci were shaken at 37° until lysed. Samples were removed at intervals, chilled to stop phage and enzyme development, and the cocci centrifuged down at low speed. To collect the intracellular materials, the chilled infected cocci were exposed to a lysing medium, composed of non-plaque-forming phage 14(145) (inactivated by ultraviolet irradiation) at about 10–20 particles/coccus and a 1/50 dilution of 14(145) phage-free virolysin. The lysates were spun down at 4000 rev./min. and the supernatant fluids were analysed for phage and lysin content. Autolysin was distinguished from virolysin by the use of autolysin antiserum.

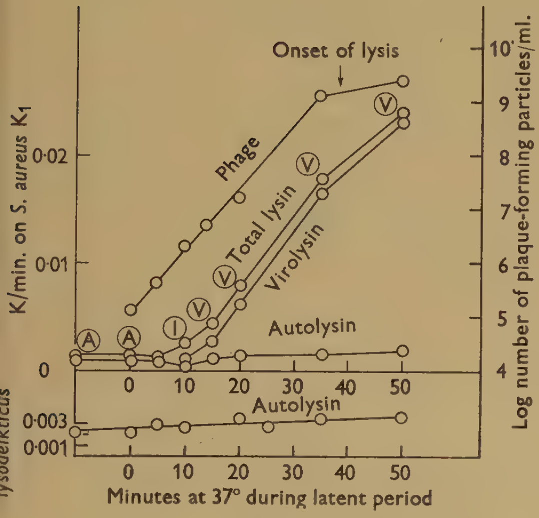

Fig. 1

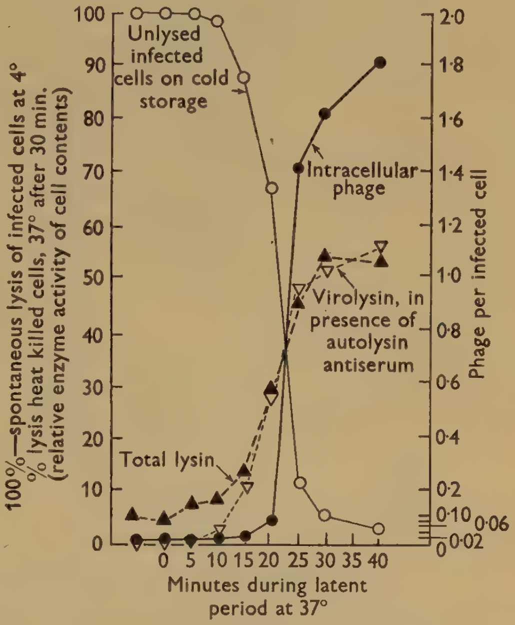

Fig. 2

Fig. 1. Formation of virolysin and phage during infection of staphylococcus strain K_1 organisms by phage P_1. The intracellular autolysin was determined from: (1) the total activity of the lysed coccal contents for *Micrococcus lysodeikticus*; (2) the residual activity of the lysates for strain K_1 cocci after exposure to virolysin antiserum. The circled letters, A, I and V, above the values for the total lysin indicate that the pH optimum of the sample was characteristic of autolysin (A), virolysin (V), or had an intermediate value (I). The phage was added at 0 min.

Fig. 2. Formation of virolysin and phage during infection of strain K_1 cocci with phage P_{14}; spontaneous lysis of infected cocci on storage in the cold. The virolysin content was determined by the residual activity of the lysates for strain K_1 cocci after treatment with autolysin antiserum. The phage was added at 0 min.

Lysin formation. Figures 1 (Expt. 1) and 2 (Expt. 2) show that a low value of lysin was present in uninfected cocci, and at 0–10 min. in a 40–55 min. latent period at 37°. This lysin was identifiable as autolysin. The autolysin content of uninfected cocci remained essentially constant (in a few experiments it increased slightly, but never as much as twofold). Material identifiable as virolysin first appeared at 10–15 min., and then it increased essentially linearly until the end of the latent period. Figure 1 shows that during the latent period a change in the pH optimum of the total lysin occurred, from that for autolysin (pH 6·5) to that for virolysin (pH 7·5).

Tests were made for possible intracellular inhibitors or activators of the enzymes. The lysed cell contents of samples of infected cocci removed at various times in the latent period were mixed with each other at final dilutions of 1/5 in TP broth. Their activity indicated that under these conditions there was nothing present at any time which interfered with any lysin present, or which activated any unknown enzymes (Table 3, Expt. 1).

Table 3. *Lack of enzyme inhibition or enhancment by mixtures of coccal contents during latent period of phage multiplication on lysin activity of individual samples*

Individual samples (time during latent period)	Enzyme activity*
Uninfected cocci	1
0 min. infected cocci	1
10 min. infected cocci	3
20 min. infected cocci	8
35 min. infected cocci	11
Phage lysate	17

Mixture of samples	Enzyme activity	
	Theoretical†	Observed
Uninfected cocci + lysate	18	20
0 min. + 20 min.	9	9
0 min. + lysate	18	19
10 min. + 20 min.	11	11
10 min. + lysate	20	22
20 min. + lysate	25	27
35 min. + lysate	28	26

* Enzyme activity = number of Klett units lysed after 48 hr. at 4°. Input in each tube was 9×10^8 heat-killed strain K_1 cocci (equivalent to 90 Klett units)/ml. Each tube contained a 1/5 dilution of samples; where a mixture was used, a 1/5 dilution of each lysin was present. Mixtures were allowed to remain at room temperature for several hours before assay cocci were added. Samples were additive.

† Sum of two individual samples calculated from above.

Relation between phage formation and enzyme synthesis. Analysis of the contents of cocci removed at intervals in the latent period indicated that the first mature particles appeared within 6 min. and then increased logarithmically until shortly before mass lysis (Fig. 1). The first detectable virolysin generally appeared somewhat later and increased linearly (Fig. 1). The phage assay was much more sensitive than the virolysin assay. With the phage assay, one mature particle could be detected, provided that all free phage was removed after adsorption. We estimate that with the enzyme, under the present conditions of assay, approximately 5×10^6 to 1×10^7 C/ml. must have formed enzyme maximally before its activity can be measured. The first detectable quantity of enzyme/ml. coincided roughly with the time at which 5×10^6 to 1×10^7 P/ml. had appeared in the disrupted cell contents, suggesting that perhaps enzyme appearance is correlated with phage maturation. However, in the absence of knowledge about the distribution of enzyme and phage among individual cocci, this correlation may be more apparent than real.

Absence of virolysin from abortive phage + coccus complexes and from uninfected cocci

While it is clear that virolysin is associated with active phage production, it would be of interest to ascertain whether it is produced by phage-adsorbed cocci which do not form virus, as well as by uninfected cocci grown and lysed under a variety of conditions.

Phage + coccus interactions. (1) *Experiments with phage-sensitized cocci.* It was found (Ralston *et al.*, 1957*a*) that when enough phage particles were adsorbed, the cocci failed to form infective centres (plaques) on agar. They did not lyse by themselves when incubated in broth, but were lysed by externally added virolysin, in contrast to uninfected or phage-producing cocci. The lysates produced in this manner did not contain phage. Cocci in this condition have been called 'sensitized'.

The following experiment was designed to test whether virolysin production could occur in phage-sensitized cocci. Staphylococci of strain K_1, grown for 4 hr. on TP agar at 37°, were resuspended in TP 2 × broth to $2{\cdot}0 \times 10^8$ C/ml. and infected with phage $P_{14}(K_1)$ at phage:coccus ratios from 50 to 2. (The addition of increasing amounts of phage to samples containing a constant number of cocci resulted in increasing numbers of sensitized cocci.) The numbers of infective centres were determined by removing samples at 20 min., treating with phage antiserum for 5 min., and then diluting for plaque count. The numbers of sensitized cocci were estimated from total phage-adsorbed cocci minus numbers of infective centres. The phage + coccus mixtures were incubated for 70 min., by which time complete lysis had occurred in all tubes. The virolysin content of the tubes was determined by assay on heated K_1 staphylococci in presence of antiserum to normal cell autolysin. As shown in Table 4, the yield of virolysin was inversely proportional to the number of sensitized cocci, suggesting that these cocci did not form virolysin. It is presumed that their lysis was brought about by the action of external virolysin released from the cocci which had undergone active infection.

Table 4. *Lack of staphylococcal virolysin production by phage-sensitized staphylococci*

Phage input per coccus	Autolysin and virolysin (total lysin) A	Autolysin (total lysin and virolysin antisera) B	Virolysin A–B	No. of cocci with infective centres*	Relative enzyme yield per infective centre†
		K/min. value			
50	0·0023	0·0012	0·0011	2×10^7	0·0012
25	0·0019	0·0006	0·0013	2×10^7	0·0010
10	0·0058	0·0012	0·0046	6×10^7	0·0010
5	0·0140	0·0035	0·0105	$1{\cdot}4 \times 10^8$	0·0010
2	0·0170	0·0058	0·0112	$1{\cdot}2 \times 10^8$	0·0014

* Total input of cocci = $2{\cdot}0 \times 10^8$/ml.

† Calculated from relative *K*/min. ÷ number infective centres × 10^7.

The conclusion that no virolysin was formed in sensitized cocci is supported by other experiments in which cocci in the logarithmic growth phase were treated with large numbers of phage particles so that virtually the entire population was sensitized. The cocci were incubated at 37° for twice the normal latent period, and at intervals samples were lysed-from-without by the addition of trace amounts of virolysin. The cell contents were then tested for lysin yield. Under these conditions, a constant amount of lysin was obtained from cocci lysed at each interval, and this was identified as normal cell autolysin.

(2) *Experiments with host-altered phage.* It was previously shown (Ralston & Krueger, 1952, 1954) that a host-controlled alteration occurred with phage P_{14}. Its host range was restricted after passage through staphylococci of strain 145 so that 34 out of 35 particles absorbed to and killed strain K_1 cocci, produced no phage and failed to cause lysis. The following experiment was carried out to test whether such cocci which did not produce phage formed virolysin (Fig. 3).

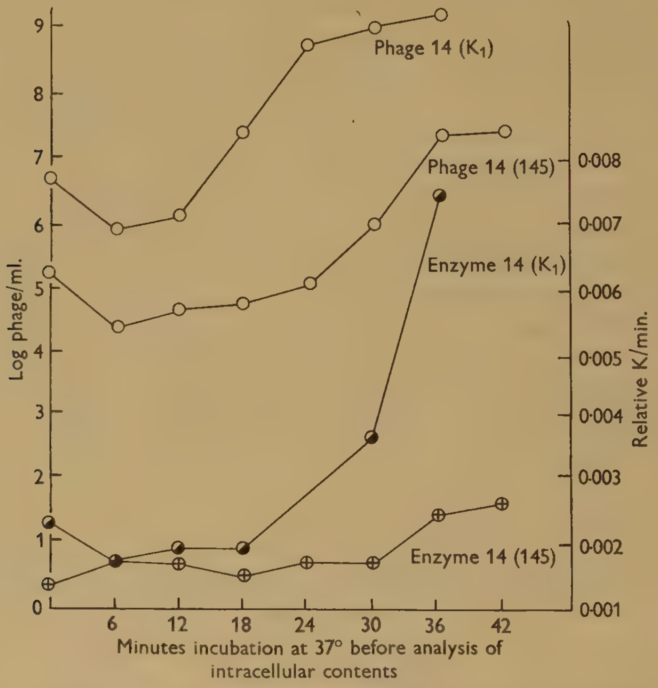

Fig. 3. Phage and enzyme formation by normal and host-altered phage P_{14}.

Strain K_1 cocci were grown on TP agar at 37°, harvested after incubation for 3 hr. (logarithmic phase) and mixed with phage P_{14}, passed previously on host strain K_1 or strain 145 at 9×10^8 P/ml. and $3{\cdot}5 \times 10^8$ C/ml. After 30 min. at 4° the temperature was raised to 37°. Samples were removed at intervals and chilled to stop infection. The intracellular contents were lysed-from-without at 4° by suspending the cocci in samples of a lysing medium as described in Expt. 2. The tubes were then assayed for their relative amounts of phage and virolysin.

In contrast to infection of strain K_1 cocci with phage 14(K_1) the contents of cocci exposed to phage 14(145) (the restricted form) yielded low amounts of virolysin and phage. The low amount of enzyme formed by this infection mixture was accounted for on the basis of the 1 in 35 cocci which produced infective centres. We conclude, therefore, that the remainder of the cocci produced no enzyme.

Attempts to find virolysin in uninfected cocci disrupted in different ways. We previously reported (Ralston *et al.* 1957*b*) that we were unable to detect virolysin in autolysates of strain K_1 cocci grown for 4 hr. and 24 hr. on TP agar and TP broth and autolysed by storage at 4° or by incubation at 37°, under toluene. In the present work further efforts were made to detect virolysin in normal cocci by extending the range of conditions and ages under which uninfected strain K_1 cocci were grown and lysed. These included growth for 1 to 48 hr. on TP agar and TP broth at temperatures from 20° to 37° and at varying rates of shaking. The cultures, ranging from 1×10^8 to 4×10^9 C/ml., were lysed in the following ways: (1) under toluene at 0° and 20°; (2) by adding tyrothricin at 0°–37°; (3) by storing cocci in shallow layers and in tall cylinders at 0°–20°; (4) by disrupting cocci with glass beads; (5) by lysis-from-without by irradiated phage and a high dilution of virolysin; (6) by shaking slowly at 35°; this results in spontaneous lysis at 6 hr. (the late exponential phase of growth). Whenever a lysin was detectable in such lysates, it was identified as normal cell autolysin.

Function of virolysin

We have suggested that virolysin releases phage by acting on a substrate forming the framework of the cell wall (Ralston *et al.*, 1955 *b*, 1957 *a*). Isolated staphylococcal cell walls, prepared by disintegrating cocci with glass beads, or by trichloroacetic acid extraction and trypsin treatment (Hancock & Park, 1958), are, in fact, dissolved by the action of the enzyme (data to be published). Further evidence to support the view that virolysin is an essential component of phage release is as follows.

Effect of inhibitors on lysis at the end of the latent period. Certain chemicals inhibit the action of external virolysin on phage- and heat-sensitized cocci (Ralston *et al.* 1957 *a*, *b*). Since there is a high concentration of virolysin in the coccus at the end of the latent period, these compounds should also inhibit the enzyme if it lyses the infected cocci-from-within (at least if the inhibitor penetrates the coccus). This was shown for $CuSO_4$ (Ralston *et al.*, 1955 *b*). In the present work, 22 other compounds, already tested for their action on external virolysin, were tested with infected cocci as follows. Strain K_1 cocci grown on TP agar for 18 hr. were mixed with phage at a phage:coccus ratio of 3:1. Samples (4 ml.) were dispensed into test tubes suitable for reading in a Klett colorimeter. The latent period was approximately 67 min. Just before the 64th min., 1 ml. solution of substance to be tested as inhibitor was added to each tube. Readings were followed turbidimetrically for a further 50 min. at 37°, and the tubes then placed at 4° overnight. The amounts of lysis of infected cocci and of phage release were then determined. Table 5 shows that for each compound, with the exception of virolysin antibody, there was a good correlation between the inhibition of external virolysin and the inhibition of lysis of infected cocci and release of phage. In the case of virolysin antibodies, unlike the other inhibitors (low molecular weight compounds), it is presumed that the antiserum cannot pass across the cell wall or membrane.

Correlation between intracellular lysin content of infected cocci and their rate of spontaneous lysis. Cocci in which virus multiplication had been interrupted by chilling lysed spontaneously on storage at 4°. Figure 2 shows the amounts of lysis after 24 hr. of cocci chilled at intervals during the latent period. Cocci removed early lysed slowly, whereas those removed at later times lysed progressively faster.

Table 5. *Effect of enzyme inhibitors on lysis of phage-infected staphylococci and on phage yield*

Compounds	Final concentration in TP broth	Action on extracellular virolysin*	% lysis of infected cocci when added at end of latent period	Yield of virus/ml.
Na citrate	0·003 g./ml.	None	80	N.t.
NaN_3	0·001 M	None	84	N.t.
$NaAsO_2$	0·001 M	None	95	1×10^8
NaF	0·001 M	None	83	$4·5 \times 10^8$
Thiourea	0·001 M	None	90	$4·6 \times 10^8$
Semicarbazide	0·001 M	None	80	$4·3 \times 10^6$
K oxalate	0·001 M	None	90	N.t.
Na sulphite	0·001 M	None	90	N.t.
KI	0·001 M	None	87	N.t.
Na deoxycholate	0·001 g./ml.	None	90	N.t.
Sulphanilic acid	0·001 M	None	80	$2·5 \times 10^8$
$PbCl_2$†	0·0004 M	Variable	26	$1·2 \times 10^8$
$K_3Fe(CN)_6$†	0·0033 M	Variable	60	1×10^7
Iodoacetic acid†	0·001 M	Variable	80	N.t.
$HgCl_2$	0·001 M	Inhibitor	0	1×10^4
$AgNO_3$	0·001 M	Inhibitor	0	6×10^6
HCHO	0·001 g./ml.	Inhibitor	0	1×10^2
Duponol	0·001 g./ml.	Inhibitor	0	1×10^6
Thiomersalate	0·001 M	Inhibitor	3	3×10^7
Hg acetate	0·001 M	Inhibitor	0	8×10^3
I_2 in KI	0·01 N in 0·25 % (w/v) KI	Inhibitor	0	1×10^1
Antibody to virolysin	1/25 dilution	Inhibitor	80	N.t.
Control	—	—	80	5×10^8

* Results from Ralston *et al.* (1957*b*) effect of various compounds on lysis of heated or phage-sensitized cocci by virolysin.

† In tests of the action of the soluble enzyme virolysin on heated cocci it was found that the degree of lysis in the presence of these compounds varied from experiment to experiment. One of the important factors appeared to be the age of the organisms used.

N.t. = not tested.

The coccal contents of the early samples yielded no demonstrable virolysin and those of later samples contained increasingly greater concentrations of it. Although the lysis of early samples was accelerated by adding the lysing medium described in Expt. 2, or by the use of tyrothricin at 4° or 37°, no increased amounts of enzyme could be recovered. These facts suggest that intracellular virolysin is not only responsible for the lysis of phage-infected cocci whenever the infection is prematurely interrupted, but also when it is allowed to proceed for the entire latent period.

DISCUSSION

Virolysin appearance in the infected coccus: new synthesis or unmasking of previously formed host enzyme? The available evidence points to the conclusion that the appearance of virolysin represents new enzyme formation following infection. This would follow if (1) the genes directing the synthesis of virolysin are inserted into the coccus at the time of virus penetration (if this proves to be the case, this system should be a favourable model for studying how genes direct protein synthesis); or (2) the phage introduces an inducer material for a repressed bacterial gene (Pardee, Jacob & Monod, 1958).

Virolysin appearance does not seem to be an activation of a previously formed host enzyme, such as is the case with DNAase in the T_{2r+} and T_{6r+} infections of *Escherichia coli* B (Kunkee & Pardee, 1956; Kosloff, 1953) for the following reasons: (1) DNAase appeared in uninfected organisms on storage, by adding a de-inhibitor, or by changing the pH value; no virolysin has been demonstrated by these devices. (2) Uninfected bacterial extracts in the T_{2r+} system produced inhibition of the DNAase activity of lysates whereas, with our staphylococci, mixing uninfected extracts with samples at different times in the latent period produced no inhibition of virolysin activity. (3) The amount of activated DNAase in the uninfected organism was greater than that in the lysate at the end of the infection cycle; we found no virolysin in uninfected staphylococci but only a low concentration of autolysin which remained constant during infection. Also,there was no antigenic fraction in uninfected cocci which produced neutralizing antibody against virolysin.

A general mechanism for the exit of viruses from host cells. Production of mucopolysaccharide-destroying enzymes may be a general mechanism evolved by viruses to accomplish their release from host cells; phage-induced lysins are known to exist in a taxonomically well-distributed number of bacterium + bacteriophage systems. In several instances it has been shown that they dissolve the bacterial cell wall which is known to contain a rigid framework of mucopolysaccharide. Similarly, the neuraminidase associated with the influenza particle may act to release the virus at the end of the growth cycle in animal cells (Gottschalk, 1958). Viral lysins may also act in penetration of the host cell by the virus particle (Adams & Park, 1956). Since our phage does not cause lysis-from-without and since the virolysin is physically distinct from phage, there is no positive evidence in our system that the lysin acts to effect entrance of the virus into the host. Moreover, other experiments (to be published) indicate that the receptor material is distinct biochemically from the mucopolysaccharide affected by the virolysin. This would suggest that the entrance of phage DNA from one particle into the cell may be accomplished by an entirely different mechanism than the exit of numerous intact particles from the cell. On this basis one might expect the particles to contain a separate enzyme responsible for destruction of a non-rigid wall component (Murphy, 1960).

Factors other than virolysin which are essential to the lysis of phage-infected cocci. The staphylococcal cell wall is resistant to high internal concentrations of lysin as well as to externally added virolysin until the end of the latent period; this suggests that a sensitizing reaction is also necessary for internal virolysin to act. Perhaps sufficient phage particles on the inside can accomplish sensitization just as they do on the outside; however, the addition of large numbers of phage particles does not produce lysis. Both external virolysin and phage are required to lyse infected cocci. These preliminary observations suggest that both virolysin and phage are 'packaged' inside the cell and are prevented from carrying out their functions until an additional mechanism releases them. While virolysin appears to be a key lytic agent, this does not exclude autolysin; but since the latter is present in lesser concentrations, this may play a minor role.

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A Protein Factor in the Nutrition of *Paramecium caudatum*

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SUMMARY

The ciliate *Paramecium caudatum* was cultivated in a medium consisting principally of known chemical compounds, including 17 amino acids, guanylic, adenylic, cytidylic and uridylic acids, sodium acetate and sodium pyruvate, linoleic and oleic acids, a mixture of B vitamins and several inorganic salts. In addition it was necessary to add microgram quantities of a protein concentrate, first obtained from autolysed yeast, but recently by an improved method from dried green peas. Lipids were first extracted from the crude material and the protein was then dissolved and precipitated with 10% trichloroacetic acid. This protein was further purified by paper chromatography to yield a concentrate active in dilutions as low as 10 μg./ml. When the protein was hydrolysed enzymically or by acid or alkali, the hydrolysates were inactive. Sixteen amino acids were qualitatively identified in the hydrolysate. The nutritional role of a protein effective in such small concentrations has not yet been satisfactorily explained.

INTRODUCTION

The ciliate *Paramecium caudatum* has many advantages for microbiological experiments, but a serious limitation on its usefulness has been the difficulty involved in maintaining pure cultures. This became apparent when Glaser & Coria (1933) first succeeded in establishing bacteria-free cultures of this species in a medium containing dead yeast cells, liver extract and kidney tissue. Shortly afterwards the rapid progress in the cultivation of smaller ciliates, particularly *Tetrahymena pyriformis*, led to comparative neglect of the genus *Paramecium* until Johnson & Baker (1942) reported the growth of pure cultures of *P. multimicronucleatum* in a special yeast medium. Van Wagtendonk & Hackett (1949) were successful with cultures of *P. aurelia* in another medium containing yeast. Burbanck (1950) showed that *P. caudatum* was similar in its growth requirements, although his medium contained killed bacteria as well as autolysed yeast. After the development of a chemically defined medium for Tetrahymena by Kidder & Dewey (1951) modifications of that medium were adapted to the requirements of Paramecium by Van Wagtendonk and his students (1953) and by Johnson & Miller (1956), but it was still necessary to add the unknown factor from yeast.

In this laboratory Sterbenz (1956) confirmed the expectation that the media developed for related species would also support growth of *Paramecium caudatum*. It appeared that the three species had a common requirement for the special factor obtained from yeast. Ordinary yeast extracts were not satisfactory and the most important part of the preparation involved a period of autolysis for several hours; when this step was omitted no growth was obtained. After such autolysis, however, the medium could be autoclaved without loss of growth-promoting activity. Although some progress had been made in purifying the unknown factor from yeast a search for another source of the factor appeared worth while. Earlier Van Wagtendonk & Hackett (1949) had reported that a lettuce infusion inoculated with *Aerobacter aerogenes* and incubated for 24 hr. before sterilization would support the growth of *P. aurelia.* A preliminary search was, therefore, made for various plant infusions which when inoculated with bacteria would yield the factor. The first significant advance was made when it was found that an infusion of split green peas could be used as a source of the factor and that under certain conditions the inoculation with bacteria was unnecessary. Thus it became possible to obtain the factor directly from peas and to prepare the concentrates more active than those previously obtained from the yeast autolysate (Lilly, Klosek & Hartig, 1958). This present report is concerned with attempts at further purification of the factor required by *P. caudatum* and improvement of the test medium in which it is used. While the unknown factor itself still has not been characterized chemically, highly active concentrates have been prepared and additional information has been obtained about its properties.

METHODS

Strains of *Paramecium caudatum* were collected from freshwater ponds on Long Island, New York, U.S.A., but not all of these proved adaptable to artificial media. Suitable strains were obtained by selecting individuals from cultures containing the natural bacterial flora and washing them by a modification of the Parpart (1928) technique previously published (Kidder, Lilly & Claff, 1940). Bacteria-free cultures were first maintained in a two-membered system originally described by Johnson (1946) with the flagellate *Polytomella caeca* as an associate. Later, when large numbers of Paramecium were obtained, they were introduced to the stock culture medium and washed free of the flagellates. This method proved more dependable for the isolation of new strains than did direct attempts at cultivating single specimens under axenic conditions. Stock and experimental cultures were maintained in 5 ml. quantities of medium in screw-capped Pyrex tubes (16 mm. diameter, 125 mm. long). The usual inoculum consisted of 0·5 ml. medium containing approximately 400 organisms from a stock culture. In a few experiments growth was determined by a direct count of the number of ciliates in a sample of 0·1 ml. taken by pipette from duplicate cultures. Maximum growth of 1000–1500 organisms/ml. was attained in the stock medium after 10–14 days, depending on the strain used. This method of direct counting was not practicable for the numerous routine tests of activity of the protein fractions. Evaluation of growth in such tests was based on a visual comparison of the experimental cultures with control cultures in stock medium. Test cultures exhibiting growth comparable with the maximum concentration in the controls were graded + + + +, and growth estimated at 3/4 maximum was graded + + +. Lower concentrations were graded + +, +, or −, but these were not considered significant, Lower rates of growth were sometimes observed when the ciliates required more than 14 days to reach a maximum, but this type of growth could not be compared with controls and was considered negative. Results of experimental cultures were regarded as positive when the rate of growth and final concentration were within 25% of that observed in controls. All tests were based on triplicate cultures carried through at least five transfers. Incubation temperature was 25°.

The first medium used for stock cultures was similar to that used by Van Wagtendonk & Hackett (1949). Fleischmann's Activated Dry Yeast was suspended in four times its weight of distilled water. This was first incubated for 4 hr. at 37° with frequent stirring, and then autoclaved for 20 min. at 120°. Most of the solids were removed by centrifugation and the supernatant fluid used as a crude stock medium. Complete removal of all suspended particles resulted in a decrease of the growth rate and of the final concentration of organisms. For stock cultures the supernatant fluid was not filtered. Since older preparations kept at room temperature had less activity than those freshly prepared the unused portion of the crude medium was kept frozen until needed; in this way activity could be preserved for several months.

Table 1. *Stock culture medium*

Preparation of pea infusion

1. 10 g. of dried green split peas suspended in 100 ml. of distilled water.
2. Boil for 15 min.
3. Remove solids by centrifugation or filtration.
4. Inoculate with one loopful of *Aerobacter aerogenes*.
5. Incubate 16 hr.
6. Autoclave at 125° for 15 min.

Preparation of medium

Pea infusion (as prepared above)	50 ml.
Proteose peptone (Difco)	5·0 g.
Cytidylic acid	0·05
Guanylic acid	0·05
Glucose	5·0
Stigmasterol	0·002

Bring volume to 1 l. with distilled water. Adjust pH to 7·0

Later, by substituting the pea infusion for the yeast autolysate a simpler stock culture medium was prepared as given in Table 1. *Aerobacter aerogenes* was introduced to produce an effect comparable to the autolysis of the yeast. By referring to the work done with other species of Paramecium and by testing various modifications on available strains of *Paramecium caudatum* a satisfactory combination of known chemical components with a minimum amount of purified fractions from the peas was eventually developed. This is the experimental test medium as given in Table 2.

RESULTS

Chemically defined components of the medium

The amino acids used were essentially the same as those used for Tetrahymena with the addition of tyrosine. Minor variations of concentrations of individual amino acids similar to the combinations used by other workers for other species of Paramecium did not appreciably affect growth. Omission of any single one or significant lowering of the concentration of any one resulted in cessation of growth. It appeared that as long as the unknown protein component was used with these

Table 2. *Experimental test medium*

All amounts given in μg./ml. of final medium

Chemically defined portion

L-Alanine	110	Linoleic acid	3·75
L-Arginine	206	Oleic acid	1·25
L-Aspartic acid	122		
Glycine	10	*Stigmasterol	2
L-Glutamic acid	233		
L-Histidine	87	*Calcium pantothenate	2
DL-Isoleucine	270	Nicotinamide	4
L-Leucine	244	Pyridoxal HCl	4
L-Lysine	272	Riboflavin	4
DL-Methionine	245	Folic acid	2
L-Phenylalanine	160	Thiamine HCl	12
L-Proline	250		
DL-Serine	394	Sodium ethylenediamine tetra-acetate	20
DL-Threonine	238		
DL-Tyrosine	100		2
L-Tryptophan	76	$MgSO_4.7H_2O$	40
DL-Valine	96	$(NH_4)_2SO_4.6H_2O$	10
		$MnCl_2.4H_2O$	0·1
Guanylic acid	75	$ZnCl_2$	0·02
Adenylic acid	30	$CaCl_2.2H_2O$	20
Cytidylic acid	75	$CuCl_2.2H_2O$	2
Uridylic acid	20	$FeCl_3.6H_2O$	0·5
Sodium acetate	570	K_2HPO_4	570
Sodium pyruvate	570	KH_2PO_4	570

Final pH adjusted to 7·0 with 0·1 N-NaOH

Undefined portion

* Protein factor obtained from peas 50 to 10

* The stigmasterol, the mixture of B vitamins and the protein factor were each sterilized separately and added aseptically to the other components which were mixed and sterilized together.

mixtures, precise quantitative studies on amino acid requirements would not be practicable. As the amount of unknown protein introduced was decreased, however, there was no indication that the amino acid balance was any more critical than previously. Further experiments on the optimum amino acid balance for *Paramecium caudatum* were postponed pending the results of more promising work on purification of the unidentified protein component.

Some of the purine and pyrimidine components of nucleic acid were also necessary for the satisfactory growth of *Paramecium caudatum*. As reported for other species the best mixture tested contained the four nucleotides, guanylic, adenylic, cytidylic and uridylic acids. The optimum proportions of these were not readily determinable while changes were constantly being made in the protein component and, as in the case of the amino acids, it was decided to use the mixture which had given the most satisfactory results in the earlier work.

An additional carbon source was required for the best growth. Glucose was satisfactory in preliminary experiments, but later sodium acetate + sodium pyruvate gave slightly better results. A sterol requirement similar to that reported for the other species was satisfied by the inclusion of stigmasterol in the medium. The

B vitamins were also required in approximately the same concentrations as reported for the other species. A mixture of inorganic salts similar to that used for Tetrahymena was included. The chelating agent sodium ethylenediamine tetra-acetate, although not necessary, was useful in preparing the stock solution of inorganic salts.

The only components of the chemically defined portion of the medium that differed much from those used for the other species were the two fatty acids, linoleic and oleic acid. After some preliminary work with natural oils, particularly corn oil, it was found that these two fatty acids in a three to one ratio promoted the best growth of *Paramecium caudatum.* The fact that in the early work with all three species the yeast autolysates contained both lipids and proteins probably contributed to some of the difficulties encountered at that time. In any event, after the lipids were separated from the pea material by preliminary extraction and replaced in the final medium by the known mixture of fatty acids it was possible to use much less of the chemically undefined material as a source of the protein factor.

Concentration of the protein factor

Progress in purification of the unidentified factor has resulted mainly from several improvements over previous methods. The first promising modification was the use of trichloroacetic acid (TCA) to precipitate the protein. This was originally used with the yeast autolysate when that material was the only source of the factor. Later this protein precipitant was found to be equally effective when the green peas were used as the source. Other methods of separating protein from non-protein material yielded preparations which were active only in much higher concentrations. There was some indication that treatment with TCA actually improved the yield of active material. When some of the native protein was treated with hydrochloric acid a precipitate was obtained that had no activity. When, however, this was dissolved in distilled water and a precipitate obtained with TCA this material proved to be active. This suggested that the native protein either contained an inactive form of the factor or that some sort of inhibition was involved. Another fortunate development coincident with the use of TCA with the crude pea infusion was the finding that no period of enzymic action or autolysis was necessary as had previously been the case when yeast was used. Removal of the TCA was carried out by dialysis against running water since the active factor was non-dialysable. The resulting product was a slightly hygroscopic white powder which could be further purified. Figure 1 outlines the method of preparation. The most active concentrates tested so far were obtained from this material by descending chromatography on Whatman no. 1 paper with 1 % (v/v) ammonia (sp.gr. 0·900) in 50 % (v/v) aqueous ethanol. Under these conditions the R_F value was 0·84. Larger quantities of concentrates were obtained by use of the same system as applied to the chromatopile described by Mitchell & Haskins (1949). When used with the chemically defined portion of the medium the concentrate was active in dilutions of 10 μg./ml.

Before chromatography the preparation, even though active in dilutions of 20 μg./ml., probably contained considerable amounts of impurities. Ultraviolet and infrared absorption spectra of this relatively crude material did not reveal any characteristic patterns of maximum absorption. After chromatography, however,

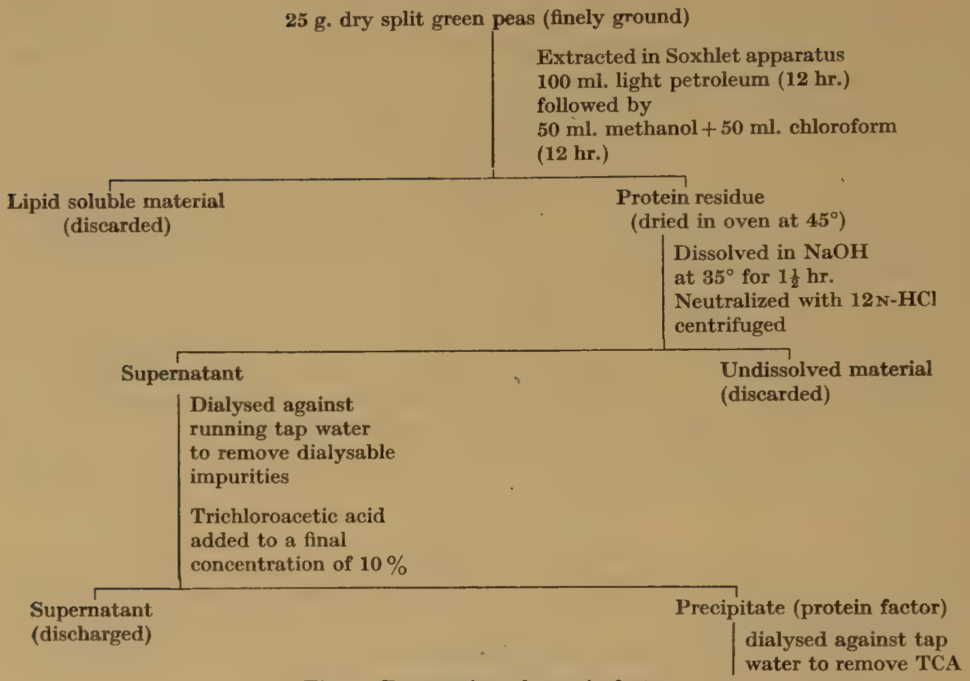
25 g. dry split green peas (finely ground)
Extracted in Soxhlet apparatus
100 ml. light petroleum (12 hr.)
followed by
50 ml. methanol + 50 ml. chloroform
(12 hr.)
Lipid soluble material
(discarded)
Protein residue
(dried in oven at 45°)
Dissolved in NaOH
at 35° for 1½ hr.
Neutralized with 12N-HCl
centrifuged
Supernatant
Dialysed against
running tap water
to remove dialysable
impurities
Trichloroacetic acid
added to a final
concentration of 10%
Undissolved material
(discarded)
Supernatant
(discharged)
Precipitate (protein factor)
dialysed against tap
water to remove TCA

Fig. 1. Preparation of protein factor.

the active material did yield reproducible spectra showing maximum absorption at 2660 Å. in the ultraviolet and more consistent patterns in infrared spectroscopy. Apparently some of the impurities contributing to the non-specific absorption had been eliminated by the chromatography.

Since all the evidence indicated that the active principle was protein in nature some experiments were done to gain information about its constitution. Firstly, most of the proteolytic enzymes, including pepsin, chymotrypsin, trypsin, papain and ficin, when allowed to act for a sufficient length of time destroyed all growth-promoting activity of the material. All enzyme preparations used were obtained from the Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A. In the experiments with pepsin 3 mg. of the commercial preparation were dissolved in 3 ml. of 0·01 N-HCl. The mixture was incubated 24 hr. at 37°. The pepsin was then inactivated by boiling and the solution evaporated to dryness *in vacuo*. In the experiment with chymotrypsin 23 mg. of the commercial preparation were added to 50 ml. of a solution containing 500 mg. of the pea protein at pH 7·8. After incubation at 25° for 24 hr. the solution was brought to pH 3 by the addition of HCl and evaporated to dryness *in vacuo*. In a similar test 12 mg. of crystalline trypsin was dissolved in 12 ml. of water and added to a solution of 250 mg. of the pea protein in 25 ml. of water at pH 7·5. In this case the incubation temperature was 37°. The above methods for the three common proteolytic enzymes were based on those described by Sanger & Tuppy (1951).

In the experiments with papain and ficin the methods were based on those published by Petermann (1946), and by Kimmel & Smith (1957). A stock solution of 200 mg. of the commercial enzyme in 25 ml. of 0·9% NaCl solution was first prepared. To 2·5 ml. of this stock preparation 1 ml. of 0·5 M-cysteine solution and 1·5 ml. of 0·1 M-sodium citrate buffer (pH 6) were added. Of this enzyme+cysteine solution 1 ml. was added to 120 mg. of the pea protein dissolved in 30 ml. of water and buffered with 0·3 ml. of the same sodium citrate solution used above. The mixture was incubated for 24 hr. at 37°.

When the active material was subjected to acid or alkaline hydrolysis the activity progressively decreased as the hydrolysis continued. After complete hydrolysis when the biuret test was negative, there was no growth-promoting activity. Acid hydrolysis was accomplished by refluxing one part of the protein with 5 parts of 6 N-hydrochloric acid for 24 hr. The hydrochloric acid was then removed by distillation under reduced pressure. Alkaline hydrolysis was carried out by refluxing one part of the protein with five parts of 6 N-barium hydroxide for 10 hr. The barium was removed with sulphuric acid.

The hydrolysates thus obtained were analysed chromatographically for their amino acid content by the two-dimensional methods described by Redfield (1953). The following were qualitatively identified: alanine, arginine, cystine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine. No evidence was obtained for the presence of either aspartic or glutamic acid either by the above methods or by the one-dimensional methods described by Hardy, Holland & Nayler (1955). Otherwise the protein involved appeared to be complete and not unusual in its amino acid composition. The possibility that more than one type of protein might have been present in the original sample of concentrate before hydrolysis was not eliminated by this preliminary study.

DISCUSSION

The fact that *Paramecium caudatum* is so common in nature suggested the extension of nutritional studies to this species. Up to the present no striking differences have been found from the known requirements of related species. The chief advance has been the discovery of a new and more convenient source of the unknown protein factor which has been the principal obstacle to formulation of a completely defined medium. Now that it is possible to obtain from dried green peas a highly active protein material by relatively simple chemical methods and without preliminary enzymic processing, more rapid progress can now be expected leading to the identification of this factor. It should be emphasized that it is by no means established that the protein obtained from the peas is necessarily identical with the material previously obtained from autolysed yeast, or that the active component of either of these artificial products enters into the nutrition of Paramecium under natural conditions. In the present state of knowledge it is difficult to conceive of a protein or a polypeptide having a vitamin-like activity, yet these latest preparations are active in microgram quantities. One possibility that should not be overlooked is that the difficulty in developing a defined medium may be due to some imbalance of the known components so that some type of toxicity or inhibitory effect on growth occurs. The role of the unknown component may be to detoxify the medium or to annul this inhibition. It is conceivable that several different types of compounds might have such an effect, comparable to the 'protective action of proteins' in other situations. In any event, even if one such compound could be isolated in pure form and its structure determined this would be a step towards understanding the mode of action.

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Some Aspects of the Genetics of Methionineless Mutants of *Salmonella typhimurium*

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SUMMARY

Forty-six methionineless mutants of *Salmonella typhimurium* were arranged in three phenotypic groups according to their growth responses to potential precursors of methionine. The results of syntrophism tests led to the recognition of two more phenotypic groups and permitted the arrangement in sequence of the metabolic steps in which mutants of each of the five groups were deficient. Transduction experiments indicated that each of these groups comprised mutants whose sites of mutation were closely linked within a complex locus. Attempts to map the sites of mutation of 12 mutants within one locus were unsuccessful. One of the mutants, probably a deletion, failed to recombine with the other 11 and behaved differently from them in linked transduction. A group of three and one pair of the 11 mutants could not be separated by recombination. Linkage was detected by transduction between only 2 of the 5 loci; these were concerned with non-sequential steps in the biosynthesis of methionine. No linkage was detected between the methionine loci and any of a number of other loci, including those controlling the biosyntheses of cysteine and tryptophan. These results were only partly in accordance with a previously suggested linkage map.

INTRODUCTION

The transfer of genetic fragments between strains LT-2 and LT-7 of *Salmonella typhimurium* and their derivatives by the temperate phage PLT-22 (transduction) was first reported by Zinder & Lederberg (1952). This was later defined as a system of common transduction in that, apparently, any marker could be transferred (Clowes, 1960). Since 1952, this system has been applied to the study of fine genetic structure by Demerec and his co-workers who used large numbers of auxotrophic, fermentative and antibiotic-resistant mutants. The genetic subdivisibility of the functional gene (locus) has been demonstrated (Hartman, 1956; Demerec & Hartman, 1956). Genes concerned with related functions were often found to be closely linked (Demerec, 1956*a*). A complementation test (abortive transduction) has permitted the definition of a functional unit (Demerec & Ozeki, 1959). Studies of the fine structure of certain loci have led to the construction of detailed linkage maps revealing, for example, the probable occurrence of deletions (Demerec, Blomstrand & Demerec, 1955; Clowes, 1958; Hartman, Loper & Šerman, 1960) and leading to an understanding of the nature of suppressor activity (Yura, 1956*b*; Gots, 1956; Howarth, 1958). Evidence has also been obtained that recombination occurs in transduction by crossing-over (Demerec & Demerec, 1955) and a copy-choice model has been suggested (Demerec, Goldman & Lahr, 1958). Fragmentation of the donor chromosome in the production of fragments incorporated into transducing phage is probably not random (Ozeki, 1959).

Previous work with 32 methionineless (*met*) mutants led to the recognition of 5 loci (*metA*, *metB*, *metC*, *metE*, *metF*) based primarily on the frequency of transduction between mutants (Glover, 1955, 1956). Growth responses to precursors of methionine, syntrophism tests and chromatographic analyses of substances accumulated by representative strains indicated that each locus comprised mutants with identical biochemical properties which were different from those of mutants of the other loci (Glover, 1956, 1958). Linkage studies (Glover, 1955, 1956) led to the construction of a map in which all the *met* loci, the 4 linked tryptophan loci (Demerec & Hartman, 1956) and 4 loci controlling cysteine synthesis were linked together.

It was originally intended to examine whether each of a number of newly-isolated *met* mutants could be assigned to an appropriate *met* locus and to investigate the fine structure of one of these loci by transduction. Nineteen new mutants and 27 of those previously used were classified and the fine structure of a locus containing 12 mutants examined. Incidental to this work was a re-investigation of linkage between the *met* loci and between these and other loci.

The system of nomenclature used is that of Demerec (1956*b*) and elaborated in the *Microbial Genetics Bulletin*, 1958, No. 16. Each type of mutant is given a symbol, e.g. *met* for methionine requirement, a number according to its chronological isolation, e.g. *met-2*, and a letter (e.g. *E*) if subsequently assigned to a locus, e.g. *metE-2*. In transduction, there is a transfer of genetic fragments from one strain of bacterium (donor) from which phage is prepared, to another strain (recipient) infected with this phage. A cross is written: recipient (bacterium) (×) donor (phage), e.g. *metE-2*(×) *metC-30*, to designate the transduction resulting from the infection of *metE-2* bacterium with phage prepared on *metC-30*. The terminology is in accordance with Demerec (1956*b*). A 'gene' is considered to occupy a linear section of a chromosome called a 'locus' concerned with the control of one function. This linear section is subdivisible into units termed 'sites' separable by crossing-over. 'Allele' is defined as one of several forms in which a gene exists. Two alleles are 'non-identical' when due to mutation at different sites, 'identical' when homologous sites are involved.

METHODS

Organisms. The wild-type strain LT-2 of *Salmonella typhimurium* (Zinder & Lederberg, 1952) and 46 different methionineless (*met*) mutants of it or of other auxotrophic derivatives from it, were used (Table 1). All the mutants except *metC-30* were obtained (in the Department of Genetics, Carnegie Institution of Washington, Cold Spring Harbor, N.Y., U.S.A.), either as spontaneous or ultraviolet-induced mutants by using the penicillin selection technique (Davis, 1948; Lederberg & Zinder, 1948). *MetC-30* was a spontaneous mutant isolated by Dr N. D. Zinder (personal communication). Twenty-seven mutants (*met-2* to *met-45* inclusive) had been used by Glover (1955, 1956, 1958) in his biochemical and genetical studies. The remaining 19 strains (*met-47 to met-68* inclusive) were newly isolated.

Stock cultures of these strains were maintained as stabs in 0·7% Bacto agar in small corked tubes which had been incubated overnight, sealed with paraffin wax and then kept at room temperature. Viability was retained during 2½ years. For day to day use, strains were kept at 0–5° on nutrient agar slopes and subcultured every 6 weeks. Their nutritional requirements were tested periodically by an auxanographic technique.

Table 1. *Methionineless* (met) *mutants of* Salmonella typhimurium

Mutant	Group	Other markers	Mutant	Group	Other markers
met- 6*	*A*	.	met-45	*B*	*adeE-11*
-7*	*A*	*cysA-21*	-49	*B*	.
-10*	*A*	*cysB-12*	-66	*B*	.
-15*	*A*	.	-30	*C*	.
-18	*A*	.	-32	*C*	*adthA-2*
-19	*A*	*tryA-8*	-35	*C*	*adthA-2*
-21	*A*	*tryA-8*	-37	*C*	*adthA-2*
-22	*A*	*tryB-2*	-50	*C*	.
-40	*A*	*adthA-2*	-52	*C*	.
-43	*A*	*adeE-11*	-56	*C*	.
-44	*A*	*adeE-11*	-57	*C*	.
-48	*A*	.	-58	*C*	.
-53	*A*	.	-65	*C*	.
-54	*A*	.	- 2*	*E*	*cysC-7*
-55	*A*	.	-47	*E*	.
-16	*B*	*cysB-16*	-51	*E*	.
-17	*B*	*cysB-16*	-60	*E*	.
-20	*B*	*tryA-8*	-61	*E*	.
-23	*B*	.	-68	*E*	.
-33	*B*	*adthA-2*	-31	*F*	*adthA-2*
-34	*B*	*adthA-2*	-31r**	*F*	.
-36	*B*	*adthA-2*	-59***	*F*	.
-39	*B*	*adthA-2*	-64***	*F*	.
-41	*B*	*adeE-11*	.	.	.

ade = adenineless; *adth* = adenine and thiaminless; *cys* = cysteineless; *try* = tryptophanless; * Obtained after ultraviolet irradiation; ** an *adth*$^+$ reversion derived from *metF-31*; *** high frequency of reversion.

In all experiments incubation was at 37°. All liquid cultures were aerated either by bubbling air through them or by agitation at 60–70 rev./min. in tubes held at an angle of 30°. Sedimentation by centrifugation was at approximately 2000 *g* for 15–20 min.; washing was carried out with 0·5 % (w/v) NaCl solution or 33 mM-phosphate buffer (KH_2PO_4) adjusted to pH 7 with NaOH.

Phage. The temperate phage PLT-22 (Zinder & Lederberg, 1952) was used for transduction. Donor lysates were prepared by the infection, at a multiplicity of 0·05–0·1, of log-phase cultures of bacteria with phage initially propagated on wild-type LT-2. Incubation was continued for 6 hr. Phage was then sedimented by centrifugation at 78,000 *g* for 1 hr. The deposited phage was resuspended in $\frac{1}{20}$th volume T2 buffer (adsorption medium; Hershey & Chase, 1953) and re-centrifuged at normal speed, the opalescent supernatant fluid then being retained. The phage preparations were sterilized by shaking with $\frac{1}{10}$th volume of chloroform for 30 min. at room temperature and then removing the chloroform by bubbling with air at 37° or room temperature. The layer-plate method (Adams, 1950) was used for phage assay, two separate 1×10^{-10} dilutions of each preparation being plated in triplicate or quadruplicate. The titres of the phage suspensions ranged from 3×10^{11} to 2×10^{12} particles/ml. and were constant during many months of storage at 0–5°.

Transduction. In early experiments, phage from donors was added to overnight broth cultures of recipient strains at 37° at a multiplicity of 5. After 6 min. adsorption, 0·1 ml. samples of the mixtures were plated on selective minimal media. Phage and recipient bacterial controls were plated at the same time. The number of prototrophic colonies was counted after incubation up to at least 40 hr. The frequency of transduction obtained with *met* mutants by this method (about 1 per 10^7–10^8 donor phage particles) was low compared with that obtained with other auxotrophs of *Salmonella typhimurium.* A 10–100-fold increase in frequency of transduction with the *met* mutants as recipients was obtained by adding phage at 4×10^{10} particles/ml. to cultures in the middle of the log phase. Cultures of suitable titre were obtained by diluting overnight broth cultures 1/20 in broth, incubating for 2 hr., centrifuging and then resuspending in $\frac{1}{4}$ volume of broth. This yielded a culture containing 2–4×10^9 organisms/ml. The multiplicity of infection was therefore at least 10. In some experiments it was necessary to have plates with 100–200 transduced colonies. After samples had been removed for plating, transduction mixtures were stored at 0–5° for 24 hr. for replating, when required, using new appropriate dilutions.

Media. The nutrient medium used was either 0·8 % (w/v) Difco Beef Peptone in 0·5 % (w/v) NaCl in water or in the tryptic meat digest broth (TMB) made in this Establishment. The addition of agar at 2 % (w/v) provided a plating medium (TMA). The minimal medium (MM) was that of Davis (see Lederberg, 1950) with the K_2HPO_4 and KH_2PO_4 concentrations adjusted to 10·5 g. and 4·5 g./l., respectively. Plating medium (MA) was obtained by the addition of agar (1·5 %, w/v). The following growth factors (μg./ml.) were added as required: adenine HCl, 20; L-cysteine HCl, 80; DL-homocysteine thiolactone HCl, 100; DL-methionine, 20; thiamin (aneurin) HCl, 1; DL-tryptophan, 20; vitamin B_{12}, 0·1. Plating medium MA was occasionally enriched with 0·01 % or 1·25 % (v/v) broth (eMA and EMA, respectively).

Purity of chemicals. The purity of the preparations of DL-cystathionine HCl, L-cysteine HCl, L-homocystine, DL-homocysteine thiolactone HCl, DL-methionine and vitamin B_{12} used in the growth response experiments was tested by means of one-dimensional paper chromatography with collidine as the solvent (Dent, 1948). The preparations of cystathionine, homocystine, vitamin B_{12} and methionine were pure; those of cysteine and homocysteine thiolactone were not. The identification of three impurities besides cystine in the cysteine was not attempted. The preparation of homocysteine thiolactone contained homocystine and probably homocysteine; these may have resulted from slow hydrolysis of the thiolactone (Dubnoff, 1952).

RESULTS

The utilization of methionine precursors on the known pathway of methionine biosynthesis in *Neurospora crassa* (Horowitz, 1947), and of vitamin B_{12}, known to be utilized by some methionineless mutants of *Escherichia coli* (Davis & Mingioli, 1950) was tested by surface inoculation of all mutants to supplemented MA plates as in the normal auxanographic technique (Pontecorvo, 1949). Plates supplemented with different concentrations of cystathionine, cysteine, homocystine, homocysteine thiolactone, methionine or vitamin B_{12} were inoculated with drops, each containing about 2×10^4 organisms, from phosphate buffer dilutions of washed organisms from overnight broth cultures of each mutant, by using a multiple inoculation device (Smith, unpublished) and incubated for 66 hr. The growth responses observed were similar to those obtained by Glover (1956, 1958); his nomenclature will be used. All mutants responded to methionine; none to cysteine. Thirty-seven mutants responded to either cystathionine, homocystine or homocysteine thiolactone (*metA* + *metB* + *metC* mutants), of which 10 (later designated *metC* by syntrophism experiments), grew slowly on MA medium and were considered to be 'leaky'. Six other mutants responded to vitamin B_{12} (*metE* mutants) and three others to methionine only (*metF* mutants). Thus, on the basis of growth responses alone, three phenotypic groups were recognizable, comprising the *metA* + *metB* + *metC*, the *metE* and the *metF* mutants.

The lowest concentrations of each substance and the times of incubation for maximum growth responses were recorded. Growth responses of mutants (other than the leaky mutants) to methionine (5–10 μg./ml.), homocystine (20 μg./ml.), homocysteine thiolactone (50 μg./ml.) and vitamin B_{12} (0·001 μg./ml.) were maximal after incubation for 42 hr. and to cystathionine (50–100 μg./ml.) after incubation for 66 hr. The leaky mutants responded to methionine, cystathionine, homocystine or homocysteine thiolactone at concentrations of one-half to one-tenth of those necessary for *metA* and *metB* mutants.

The slow growth response of *metA* + *metB* + *metC* mutants to cystathionine at high concentration was further investigated by chromatographic examination of solutions of the substance in sterile distilled water and MM medium after incubation for 66 hr. The former was pure but the latter contained small quantities of two impurities, one of which was homocystine, which might well have been responsible for the growth in the presence of cystathionine. However, the lag in growth response to cystathionine still persisted after inoculation on to MA medium + cystathionine at 50 μg./ml. pre-incubated for 66 hr.

Indication of the sequence of metabolic blocks with which each group of mutants was associated

This was determined by means of syntrophism tests. Two methods gave readily reproducible results.

Parallel streaking. Loop inocula from saline dilutions (1/1000) of washed overnight broth cultures of mutants were streaked 0·5–1 cm. apart on EMA medium, each pair of inocula being isolated either in separate 4 cm. diameter Petri dishes or on sectors of solid medium in 9 cm. diameter Petri dishes. Control streaks of homologous pairs and mutants alongside wild-type were always made. Syntrophism was indicated by relatively denser growth of the edge of a streak adjacent to one releasing a diffusible compound (accumulant) and was visible after incubation for 60 hr. but more clearly after 85–90 hr.

All paired combinations of the strains *metA-6*, *B-16*, *C30*, *E-2* and *F-31*, representative of Glover's 5 groups, were tested in this way. Strain *metC-30* fed strains *metA-6* and *B-16* but not strains *metE-2* or *F-31*. No other feeding was clearly demonstrated. The other 9 leaky strains behaved like strain *metC-30* in similar experiments. These results indicated that the 10 leaky mutants comprised a fourth phenotypic group (*metC*) and that the metabolic blocks in the biosynthesis of methionine associated with the *metA* and *B* mutants preceded, and those of the *metE* and *F* mutants followed, those of *metC*.

Replica plating. Inocula (0·1 ml.) from washed overnight broth cultures of strains *metA-15, B-23, C-30, E-47* and *F31r*, were spread on single EMA medium plates (eMA medium for *C-30*) which, after drying, were stored overnight in the refrigerator. Loop inocula from each culture and a similar culture of wild-type were also spread over well-separated circular areas of about 1 cm. diameter on each of 6 TMA plates, over a template. After overnight incubation, replication was carried out (Lederberg & Lederberg, 1952) from each of these plates to single different refrigerated lawn plates and to an additional uninoculated plate. These plates were then observed during incubation for 96 hr.

Table 2. *Syntrophism of representative* met *mutants of* Salmonella typhimurium

Samples (0·1 ml.) of washed organisms from overnight broth cultures of each mutant were spread as lawns on single EMA medium plates (*eMA* medium for *metC-30*) which were refrigerated overnight. Loop inocula of each mutant and wild-type were spread over areas about 1 cm. diameter on each of 6 TMA medium plates, over a template. After overnight incubation, replication was made from each of these plates to a different refrigerated plate and to an uninoculated EMA medium plate. These plates were then incubated for at least 96 hr.

	Lawns				
Replicate areas	*metA-15*	*B-23*	*C-30*	*E-47*	*F-31r*
metA-15	−, 2	−, 6	−, 6	−, 6	−, 6
metB-23	+, 2	−, 2	−, 6	−, 6	−, 6
metC-30	+, 4	+, 2	−, 5	−, 6	−, 7
metE-47	±, 6	−, 6	±, 7	−, 1	−, 2
metF-31r	±, 6	−, 5	±, 6	−, 1	−, 1
Wild-type	−, 8	−, 8	±, 8	−, 8	−, 8

+ = presence of halos of growth *around* replicated area; − = absence of halos of growth *around* replicated area; ± = poor halos of growth *around* replicated area; 1–8, relative density of growth *within* replicated area.

Syntrophism was evident from the growth response of organisms in the surface lawns as halos around replicated areas, and from the relative density of growth within different replicated areas on each lawn (Table 2). Halos indicated the diffusion of an accumulant to which organisms in the lawns responded. Thus it was inferred that the metabolic block of strain *metA-15* preceded that of *B-23* and that both the *metA-15* and *B-23* blocks preceded that of *metC-30*. There was also a suggestion that the *metA-15* and *C-30* blocks preceded those of *metE-47* and *F-31r*. Allowing for poorer growth within areas surrounded by halos, and the leaky growth within *metC-30* areas, the relative density of growth within other areas confirmed the inferred sequence of metabolic blocks *metA-15–metB-23–metC-30*. No syntrophism of *metE-47* and *F-31r* was observed, but syntrophism of *metA-15, B-23* and *C-30* with both *metE-47* and *F-31r* did occur, although the direction of feeding was uncertain. Fourteen of the other 25 (*metA* and *metB*) mutants behaved exactly as *metA-15* and 11 exactly as *met-B23* in further similar experiments. This indicated that these mutants might be divided into two phenotypic groups, comprising 15 *metA* and 12 *metB* mutants.

To find the direction of feeding in syntrophism of *metA*, *metB* or *metC* mutants with *metE* or *metF* mutants, the relative numbers of organisms of each phenotype in replicate areas showing syntrophism of *metA-15*, *metB-23* or *metC-30* with *metE-47* or *metF-31r* was determined, assuming that fed organisms would proliferate more than feeders. Loopfuls of growth from these areas were suspended in 0·5 ml. phosphate buffer and appropriate dilutions plated on TMA medium to yield about 100 single colonies/plate. After overnight incubation replication to MA, MA + homocysteine thioloactone, MA + vitamin B_{12} (where *metE* organisms were involved) and TMA media was carried out and the plates incubated for 4 hr. at 37° and 17 hr. at room temperature.

Between 200 and 500 colonies derived from each area were screened. In all cases, colonies with the *metA*, *B* or *C* phenotype predominated. They comprised an average of 91 % of those from organisms in areas on lawns of *metA-15*, *met-B23* or *metC-30* and 82 % from areas on lawns of *metE-47* or *metF-31r*. It was concluded that the *metA*, *metB* and *metC* metabolic blocks preceded those of *metE* and *metF*.

In summary, the results of the syntrophism tests indicated the existence of 5 phenotypic groups amongst the methionineless mutants (*metA*, *metB*, *metC*, *metE*, *metF*) and that the probable sequence of the metabolic blocks in the biosynthesis of methionine was: *metA–metB–metC–*(*metE–metF*). The arrangement of the *metE* and *metF* blocks in sequence was not possible because of the lack of syntrophism of *metE* and *metF* mutants.

The relationship between phenotypic and transduction groups

This was tested by determining the frequency of transduction to produce prototrophs between all mutants of each phenotypic group and single representative mutants of other groups as recipients with appropriate single representative strains of each of Glover's (1956, 1958) five groups and the wild-type as donors. A lower frequency of transduction between mutants indicated close linkage and membership of the same transduction group (Demerec, Blomstrand & Demerec, 1955). The experiments were carried out in three stages.

The transduction groups amongst the *metA* and *metB* mutants were determined by using the original transduction technique. The selective medium was EMA. From the results recorded in Table 3 (*a*) it can be seen that relatively few if any prototrophs resulted from transduction between all 15 of the *metA* mutants and *metA-6*, or between the 12 *metB* mutants and *metB-17*, thus indicating two transduction groups.

Transduction involving all of the *metC* strains as recipients was carried out in T2 buffer instead of broth; plating was on eMA medium. This controlled residual growth of the leaky *metC* strains and made prototrophic colonies easier to count. The improved transduction technique was used. The results in Table 3 (*b*) indicated that 9 of the 10 *metC* mutants comprised one transduction group. A high frequency of reversion did not permit the use of *metC-57* as a recipient or donor.

Transduction groups amongst the *metE* and *metF* mutants were determined at the same time. Of the three *metF* mutants (Table 1), *metF-59* and *metF-64* showed high frequencies of reversion. That of *metF-59* was too high for its use as donor or recipient but that of *metF-64* (approximately 1 in $1{\cdot}25 \times 10^6$ organisms) permitted its use as a donor. The improved transduction technique was used, with plating on MA medium. All of the *metE* mutants appeared in one group and *metF-31* and *metF-64* in another (Table 3 (*c*)). The decreased frequency of transduction in the *metB-23* (×) *metF-64* cross may indicate linkage between these two mutants. Although all possible crosses between methionineless mutants of the same

Table 3. *The numbers of prototrophs obtained in transduction between* Salmonella typhimurium met *mutants of different phenotypic groups*

Each figure represents the total number of colonies obtained on three enriched MA plates (EMA or eMA media) less the total number of colonies on three recipient control plates after 41 hr. of incubation. Each plate was inoculated with about 2×10^8 organisms from overnight broth cultures (centrifuged and resuspended in T2 buffer where *metC* mutants were recipients) and 1 or 4×10^9 phage particles which had been incubated together for 6 min.

(*a*) *metA* and *metB* mutants

	Donor				Donor		
Recipient	*metA-6*	*metB-17*	Wild-type	Recipient	*metA-6*	*metB-17*	Wild-type
metA-6	0	379	211	*metB-16*	81	0	128
A-7	0	175	122	*B-17*	115	0	114
A-10	0	476	240	*B-20*	83	0	156
A-15	0	41	38	*B-23*	140	5	138
A-18	0	120	62	*B-33*	213	0	195
A-19	10	81	44	*B-34*	91	0	111
A-21	2	148	81	*B-36*	212	0	308
A-22	3	109	82	*B-39*	93	9	147
A-40	7	419	170	*B-41*	72	0	121
A-43	0	445	268	*B-45*	168	26	201
A-44	19	210	102	*B-49**	20	0	14
*A-48**	2	125	64	*B-66**	142	21	176
*A-53**	3	146	83	*C-32*	96	259	137
*A-54**	4	139	74	*E-2*	91	92	86
*A-55**	0	143	65	*F-31*	110	213	142

* New mutants assigned to their transduction groups.

(*b*) *metC* mutants

	Donor			Donor	
Recipient	*metC-30*	Wild-type*	Recipient	*metC-30*	Wild-type*
metC-30	0	3840	*metC-57*	High frequency of reversion	
C-32	590	5850	*C-58*	0	1445
C-35	207	6705	*C-65*	9	5245
C-37	72	2950	*A-15*	3554	1615
C-50	24	1725	*B-23*	4040	3050
C-52	721	3255	*E-2*	3977	2955
C-56	712	3115	*F-31*	6135	5615

* Counts of platings of 1/5 dilutions × 5.

(*c*) *metE* and *metF* mutants

	Donor			
Recipient	*metE-2*	*metF-31*	*metF-64*	Wild-type*
metE-2	0	6459	3719	4180
E-47	798	X	X	6910
E-51	38	X	X	695
E-60	295	X	X	4970
E-61	124	X	X	2310
E-68	108	X	X	5720
F-31	11010*	0	604	8710
*A-15**	7170	8630	6550	5910
*B-23**	6190	5320	1750	5800
*C-30**	3920	5010	2790	3840

* Counts of platings of 1/10 dilutions × 10.
X = no crosses made.

phenotypic groups were not made, the results of the above experiments were considered to show that the transduction and phenotypic grouping of these mutants was coincident.

Attempts to detect abortive transduction

Attempts to detect abortive transduction between representative mutants of each *met* group and wild-type were carried out at different times in several different ways. Both mid and late log-phase recipient cultures were used. Plating from T2 buffer as well as broth to MA, eMA, EMA, MA + neutralized vitamin-free casein hydrolysate at 0·2 % and MA + casamino acid at 0·1 % (Demerec *et al.* 1957) media was tested. Observations were made during incubation for 7 days, either continuously at 37° or for 1 day at 37° and then 6 days at room temperature as suggested by Dr H. Ozeki (personal communication). In no case were colonies characteristic of abortive transduction obtained (Ozeki, 1956) although Ozeki (1959) reported abortive transduction in the cross *metC-50* (×) wild-type.

Mapping the sites of mutation of the metB *mutants*

This was attempted by determining the frequency of transduction to produce prototrophs in a series of crosses between them. The improved transduction technique was used and plating was on EMA medium. The same phage preparations were used throughout and estimates of donor and recipient efficiency obtained by transductions using *cysC-7* as recipient with all the *metB* strains as donors, and all of these strains as recipients with phage from the wild-type donor strain. To facilitate counting, 1/10 broth dilutions of these control transduction mixtures were plated. From the results (Table 4) three transduction subgroups within which

Table 4. *The numbers of prototrophs obtained in transductions between* Salmonella typhimurium *mutants of the* metB *group*

Numbers as in Table 3. Plating was on EMA medium.

Recipient	Donor												
	metB												
	-16	*-17*	*-20*	*-23*	*-41*	*-66*	*-33*	*-34*	*-49*	*-36*	*-39*	*-45*	Wild-type*
metB-16	0	0	0	314	613	393	307	239	132	0	453	542	7170
-17	0	0	0	277	404	392	363	155	118	0	444	457	7040
-20	0	0	0	56	20	66	110	8	44	0	69	137	9200
-23	267	218	33	0	9	11	161	73	123	0	241	78	6300
-41	299	126	80	13	0	18	168	35	96	0	156	157	4610
-66	248	144	87	6	27	0	153	40	98	0	81	147	4930
-33	379	192	132	115	220	256	0	44	0	0	135	541	8230
-34	125	112	0	36	28	130	21	0	0	0	147	240	9220
-49	1073	466	140	322	475	622	0	35	0	0	212	856	10260
-36	0	0	0	0	0	0	0	0	0	0	0	0	1350
-39	360	179	221	159	153	158	646	67	56	0	0	484	8580
-45	1233	535	532	136	260	129	975	265	316	0	578	0	13040
*cysC-7**	14710	12570	5210	4990	7020	16880	5760	4780	4950	15250	6590	6890	13380

* Counts of 1/10 dilutions × 10.

transduction occurred at low frequency or not at all were apparent. They comprised *metB-16*, *metB-17* and *metB-20*; *metB-23*, *metB-41* and *metB-66*; *metB-33*, *metB-34* and *metB-49*, respectively. No transduction occurred between the members of the first sub-group or between *metB-33* and *metB-49* in the third subgroup. On the basis of frequency of reversion and behaviour in crosses with all other *metB* mutants, *metB-16* and *metB-17* were similar to each other but different from *metB-20*. However, *metB-33* and *metB-49* were dissimilar in these respects.

No transduction was obtained in reciprocal crosses between *metB-36* and all other *metB* mutants, and transduction between *metB-36* and wild-type occurred at a lower frequency than that between other *metB* mutants and wild-type. As a donor in crosses with mutants of other *met* groups, *metB-36* was as efficient as other *metB* mutants. No reversions were detected on *metB-36* control plates even when each of 10 plates was inoculated with 10 times the number of organisms normally plated in transduction experiments.

The construction of a linkage map from the data of Table 4 was attempted. To correct for donor and recipient efficiency, the frequency of transduction for each cross was multiplied by both the ratios of the frequencies of transduction in the crosses *cysC-7* (×) wild-type/*cysC-7* (×) donor, and *cysC-7* (×) wild-type/recipient (×) wild-type, respectively. As an initial step, linear arrangement of the three transduction subgroups was tried. From the results of an analysis of corrected data from reciprocal crosses between different sets of single representative mutants of each subgroup it was not possible to construct a linear map. The same difficulty was encountered by using the sum of reciprocal transduction between pairs of *met* strains (after Hartman, 1956).

Linkage between the metA, B, C, E *and* F *groups*

This was tested by transduction between the representative strains *metA-15*, *B-23*, *C-30*, *E-47* and *F-31* in a series of donor phenotype selection experiments similar to those used by Clowes (1958) with cyst(e)ineless mutants. If donor and recipient differ phenotypically and it is possible to detect the donor phenotype amongst large numbers of organisms of the recipient phenotype, then the frequency with which transduction between mutants results in organisms which are wild-type for the recipient marker but mutant for the donor marker, should indicate the proximity of the sites of mutation concerned.

Three donor phenotypes were potentially detectable in transduction between pairs of mutants belonging to the different *met* groups. They were: the ability to utilize homocysteine thiolactone (*met*$^{\mathrm{homth}}$) or vitamin B_{12} (*met*$^{B_{12}}$) as an alternative to methionine: attributes of *metA*, *metB* or *metC* and *metE* mutants, respectively; and leaky growth on MA medium when plated from broth (*met*$^{\mathrm{leaky}}$) coupled with feeding of *metA* and *metB* mutants, attributes of *metC* mutants. The crosses in which each phenotype might have been detected are indicated in Table 5.

The ability to differentiate colonies with the different *met* phenotypes from wild-type arising after transduction was established in a series of reconstruction experiments under the conditions summarized in Table 6. All the permissible types of crosses (Table 5) were made by using the improved transduction technique, suitable dilutions of the transduction mixtures being made to give 100–200 transduced colonies/plate. Infected recipient cultures of *metC-30* were diluted in T2 buffer rather than in broth, to avoid too heavy background growth on the selective medium. Approximately 2000 (range 1257–3025) colonies from each cross were screened for donor phenotype. Transduction of donor phenotype was detected only in the *metF-31* (×) *metB-23* cross in which 1139 out of 3025 (38 %) transduced colonies were of the *met*$^{\mathrm{homth}}$ phenotype.

In these experiments, linkage between all pairs of mutants from the different groups, except between those of *metA* and *metB*, was tested in crosses involving transduction in at least one direction. Linkage between *metA* and *metE*, *metB* and *metE* and *metC* and *metE* was tested in both directions (Table 5). It was concluded that no linkage was detectable except between *metB* and *metF*. Assuming a linear arrangement of sites of mutation and linkage of *metB* and *metF*, *metA* could either be between *metB* and *metF* or distal to *metB* on the same side of *metF* as *metB*. If the first situation prevailed, greater than 38 % linked transduction between *metA* and *metF* should have been obtained. In fact no *metA-metF* linkage was detected. Allowing for a 2 % error in the replication technique (Clowes, 1958) and assuming that the production of transducing phage did not involve fragmentation between *metA* and *metB* (Ozeki, 1959), it was concluded that, if *metA-metB* linkage existed, the distance between *metA* and *metB* was at least 19 times that between *metB* and *metF*.

Table 5. *Donor phenotypes* Salmonella typhimurium *met in mutants detectable in inter-group transduction*

Recipient	**Donor (phage)**				
	metA	*B*	*C*	*E*	*F*
metA	0	X	*met*$^{\mathrm{leaky}}$	*met*$^{B_{12}}$	X
B	X	0	*met*$^{\mathrm{leaky}}$	*met*$^{B_{12}}$	X
C	X	X	0	*met*$^{B_{12}}$	X
E	*met*$^{\mathrm{homth}}$	*met*$^{\mathrm{homth}}$	*met*$^{\mathrm{homth}}$	0	X
F	*met*$^{\mathrm{homth}}$	*met*$^{\mathrm{homth}}$	*met*$^{\mathrm{homth}}$	*met*$^{B_{12}}$	0

met$^{\mathrm{homth}}$ = utilization of homocysteine thiolactone; *met*$^{B_{12}}$ = utilization of vitamin B_{12}; *met*$^{\mathrm{leaky}}$ = leaky growth on MA and feeding of *metA* and *B* strains; X = donor phenotype not detectable; 0 = homologous crosses.

In conjunction with the previously described attempts to map the sites of mutation of *metB* mutants (Table 4) the frequency of transfer of donor phenotype in crosses between *metF-31* and each of the *metB* mutants was determined. Transduction was carried out under optimum conditions for the detection of the *metB* *met*$^{\mathrm{homth}}$ phenotype (Table 6) except that only about 1000 colonies/cross were screened for donor phenotype. An average of 27·5 % (range 21·6–34·1 %) transfer of donor phenotype was obtained in crosses involving 11 of the 12 *metB* mutants as donors. The exception was *metB-36* with which only 3·6 % transfer was obtained. These results indicated that all the *metB* sites were linked to *metF-31* but that 11 of them were clustered together closer to *metF-31* than to *metB-36*.

Linkage between met *mutants and certain other markers*

Linkage was tested by transduction in two ways.

By donor phenotype selection. This was carried out in transduction between the 5 representative *met* mutants used in the intergroup crosses above (recipients) and the six double-auxotrophs *metA-7cysA-21*, *metB-16cysB-16*, *metE-2cysC-7*,

Table 6. *Optimum conditions for the detection of the different donor phenotypes of* met *mutants of* Salmonella typhimurium *in intergroup transduction*

Donor phenotype	Recipient	Initial selection: Medium	Initial selection: Time of incubation	Replication: Media	Replication: Time of incubation	Characteristics of donor phenotype
met^{homth} (*metA*, *B* and *C*)	*metE* and *F*	EMA + homth	41 hr.	MA and MA + homth	17 hr.	Growth on MA + homth; not on MA
met^{leaky} (*metC*)	*metA* and *B*	MA	6 days	.	.	Microcolonies (1–1·5 mm. diam.) + feeding of background
$met^{B_{12}}$ (*metE*)	*metA* and *B*	EMA + B_{12}	41 hr.	MA and MA + B_{12}	17 hr.	Growth on MA + B_{12}; not on MA
	metF	EMA + B_{12}	28 hr.			
	metC	MA + B_{12}	41 hr.			

homth = homocysteine thiolactone at 100 μg./ml.
B_{12} = vitamin B_{12} at 0·01 μg./ml. (i.e. 1/10th of concentration used in growth response experiments)

metB-20 tryA-8, *metF-31 adthA-2* and *metB-41 adeE-11* (donors). The initial selective medium was supplemented with cysteine, tryptophan, adenine and thiamin or adenine where appropriate, to facilitate growth of colonies with donor phenotype. The screening of about 1000 colonies from each cross was attempted. Initial selection plates were incubated for 24–65 hr. according to the cross, until all of the transduced colonies were about the same size (1–2 mm. diameter) before replication to unsupplemented and supplemented media. The replicated colonies were observed

Table 7. *Attempts to detect the transfer of donor phenotype in transduction between representative mutants of the five* met *groups and certain other mutants of* Salmonella typhimurium

Transduction was as described in Table 3. The MA medium for each cross was supplemented with either cysteine, tryptophan, adenine or adenine + thiamin according to the additional nutritional requirement of each donor strain. The initial incubation was for 24–65 hr. After replication to unsupplemented and supplemented MA medium the plates were incubated for 17 hr.

Recipient	Donor					
	metA-7* cysA-21	metB-16* cysB-16	metE-2* cysC-7	metB-20* tryA-8	metF-31 adthA-2	metB-41 adeE-11
	No. of colonies screened without detecting donor phenotype					
metA-15	0	1197	1107	1024	1060	1034
B-23	1140	841	730	0	1047	0
C-30	603	1279	716	1056	975	1288
E-47	1355	1533	941	1132	851	820
F-31	1665	1401	1316	847	×	**

* No linked transduction between these double auxotrophs and wild-type (see Table 8).

** Not tested, since selection of donor phenotype *ade* impossible because the additional marker of *metF-31* is *adth*.

× Homologous cross.

after incubation for 17 hr. No colonies with donor phenotype resulted from any of these crosses (Table 7). Three crosses yielded no transduction and the donor phenotype could not be selected in another so that linkage between *metA* and *cysA*, *metB* and *tryA* or *adeE* and *metF* and *adthA* could not be tested. There therefore appeared to be no linkage between: *metA* and *cysB*, *cysC*, *tryA*, *adthA* and *adeE*; between *metB* and *cysA*, *cysB*, *cysC* or *adthA*; between *metC* and *cysA*, *cysB*, *cysC*, *tryA*, *adthA* or *adeE*; between *metE* and *cysA*, *cysB*, *cysC*, *tryA*, *adthA* or *adeE*; or between *metF* and *cysA*, *cysB*, *cysC* or *tryA*.

By transduction of doubly auxotrophic recipients with phage from wild-type donor

This involved the attempted transduction of both markers simultaneously. Crosses of all of the double auxotrophs used above, with *metA-19 tryA-8* substituted for *metF-31 adthA-2*, were made by the improved transduction technique. Each transduction mixture was plated on MA medium, MA + methionine medium and MA + cysteine, tryptophan or adenine medium, according to the alternative nutritional requirement of the recipient. Colonies on the supplemented media were counted after incubation for 41 hr. Final observations of all plates were made after incubation for a further 48 hr. Replication was then made from the supplemented plates to MA medium. The replica plates were observed for wild-type colonies during incubation for 48 hr. The results of these experiments (Table 8), confirmed the absence of linkage between *metA* and *tryA*, *metB* and *cysB* and *metE* and *cysC*, and also indicated its absence between *metA* and *cysA* and *metB* and *tryA* or *adeE*.

Table 8. *Attempts to detect linked transduction between certain* Salmonella typhimurium met *mutants and other loci, by the use of double auxotrophs*

Numbers as in Table 3 except that plating was on MA medium. Where the MA medium was supplemented, platings were made from 1/10 dilutions of the transduction mixtures in addition to those from the concentrated suspensions.

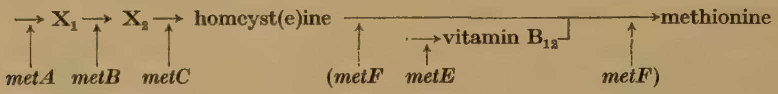
	Recipient					
	metA-7 *cysA-21*	*metB-16* *cysB-16*	*metE-2* *cysC-7*	*metA-19* *tryA-8*	*metB-20* *tryA-8*	*metB-41* *adeE-11*
Media of selection	No. of colonies resulting from transductions using wild type donor					
MA	0	0	0	0	0	0
MA + methionine	4500*	1484	3000*	9000*	14000*	1968
MA + cysteine, tryptophan or adenine	544	1214	568	3580*	4070	2765

* Approximate counts.

DISCUSSION

Metabolic blocks in a possible pathway of methionine biosynthesis

Homocysteine is believed to be a precursor of methionine in several different micro-organisms, e.g. *Neurospora crassa*, *Bacillus subtilis*, *Escherichia coli* (Young & Maw, 1958) and *Streptococcus lactis* (Kizer, Speck & Aurand, 1955) and is implicated in the synthesis in *Aerobacter aerogenes* (Shapiro, 1955). Davis & Mingioli (1950) reported methionineless mutants of *E. coli* W which showed similar growth responses and syntrophism to those of the *Salmonella typhimurium metE* and *metA*, *B* and *C* mutants, and postulated that vitamin B_{12} was a component of a coenzyme concerned with the methylation of homocysteine. This function of vitamin B_{12} seemed to be supported by studies of the synthesis of methionine with cell suspensions and cell-free extracts of at least one of these mutants (Gibson & Woods, 1952; Helleiner & Woods, 1956). The growth response of members of the *S. typhimurium metA*, *B* and *C* phenotypic groups to homocystine and homocysteine thiolactone suggested that homocystine or homocysteine (homocyst(e)ine) was also on the pathway of methionine biosynthesis in *S. typhimurium* and that these mutants were blocked in metabolic steps before this intermediate, and *metE* and *F* mutants after it. This sequence is supported by the results of the syntrophism experiments and the existence of three metabolic blocks associated with *S. typhimurium metA*, *metB* and *metC* mutants in that sequence established (Fig. 1).



X = unknown precursor of methionine; ⇡ = possible alternative positions of metabolic block; ( ) = indeterminate sequence of blocks.

Fig. 1. A suggested pathway of methionine biosynthesis in *Salmonella typhimurium*.

Since *metE* and *metF* mutants fed *metA*, *B* and *C* mutants but themselves failed to show syntrophism, it was concluded that their metabolic blocks involved inability to carry out some (probably the same) biosynthetic step between homocyst(e)ine and methionine. It is suggested that the function of vitamin B_{12} in the biosynthesis of methionine is the same in *Salmonella typhimurium* and *Escherichia coli* and that the *S. typhimurium metE* mutants lack the coenzyme concerned with the methylation of homocysteine of which vitamin B_{12} (or a derivative of it) is a component. *S. typhimurium metF* mutants may be considered to lack the enzyme necessary for this metabolic step.

The results of these biochemical studies are in general agreement with those outlined by Glover (1956, 1958) who used some of the same mutants, except that the growth response of *Salmonella typhimurium metA* and *B* mutants to cystathionine was not clearly confirmed. However, cystathionine should not perhaps be excluded as an intermediate, for Wijesundera & Woods (1953) showed that 8 out of 10 mutants of *Escherichia coli* which possessed cystathionase were able to convert exogenous cystathionine to homocysteine but only synthesized small quantities of methionine. They suggested that other enzymes might compete for homocysteine; perhaps the differences between cultural conditions for *S. typhimurium metA* and *B* mutants in Glover's experiments and those reported in this paper, affected the relative activity of these competing enzymes so that a definite growth response to cystathionine was obtained in the former but not in the latter.

Correlation of phenotypic and transduction groups

Members of the same transduction group of *Salmonella typhimurium met* mutants possessed identical biochemical characteristics so far as they were tested and each can be regarded as an allele of one of 5 complex gene loci (Demerec, 1956*b*) namely *metA*, *metB*, *metC*, *metE* and *metF*. In the absence of a complementation test (abortive transduction) it was not possible to decide whether each locus was a functional unit. Demerec & Ozeki (1959) gave examples of complex loci defined in the same way as the *met* loci but subsequently shown to comprise more than one closely linked functional unit. They also reported, incidentally, that complementation might not always serve to differentiate such loci and that abortive transduction was not detectable with some mutants.

The metB *locus*

The attempts to map the sites of mutation of the *metB* locus in *Salmonella typhimurium* were subject to limitations, because, even with the improved transduction technique, the efficiency of transduction of *met* mutants was less than that of most other mutants of *S. typhimurium* and hence the chances of recombination were decreased. The resolving power within *met* loci might well have been as low as 1% of that within the *his* locus (Hartman, Loper & Serman, 1960) and 10% of that within the *try* locus (Demerec & Hartman, 1956) respectively. It was perhaps not surprising that a linear map of the *metB* locus could not be constructed. For accurate location of sites three- or four-point linkage tests are a great advantage, especially when the efficiency of transduction is low (compare Clowes's (1958) studies of the *cysB* locus). Unfortunately, no linkage was detected between any of the *met* loci and a number of others suitable for such tests.

The properties of *Salmonella typhimurium metB-36* indicate that it is a multiple-site mutant (Demerec, 1956*a*) in that it failed to recombine in reciprocal transduction with a number of closely-linked mutants, that it was the only mutant of the 12 *metB* mutants to do this, and that it failed to revert. Definition of the region of the chromosome covered by the *metB-36* mutation is not possible at present. It does not cover the *metF* locus to which the *metB* locus is linked, but may cover an undiscovered locus (or loci) between *metB* and *metF* or on the other side of *metB*, or may even cover loci on both sides of *metB*. The failure to obtain transduction between one group of three (*metB-16*, *B-17* and *B-20*) and one pair (*metB-33* and *B-49*) of the *metB* mutants other than *metB-36* may have been due to identical-site mutation at two sites (Demerec, 1956*a*). There is evidence that some identical-site mutants show similar frequencies of reversion (Demerec, 1956*a*), and similar behaviour in intra-locus transduction might be anticipated. For *metB-16*, *B-17* and *B-20* and *metB-33* and *B-49* incidental observations of their reversion frequency and the data of Table 4 revealed that only *metB-16* and *B-17* were similar in these respects. It was concluded that the sites of mutation of *metB-16* and *B-17* might well be identical, but *metB-20* differed from these two and *metB-33* and *B-49* from each other, in that mutation in these mutants involved overlapping but probably different numbers of sites on the chromosome. The speculation could be made that each mutation in the *metB* locus other than *metB-36* (probably a deletion) is a multiple-site change involving different small lengths of the chromosome. This would perhaps explain mapping difficulties within the locus.

Behaviour of metB-36 *in transduction*

In the *metF-31* (×) *metB-36* cross only 3·6% donor phenotype transfer was obtained as compared with an average of 27·5% with other *metB* mutants as donors.

From the results of transduction between all the *metB* mutants (Table 4), *metB-36* appears to be a multiple-site mutant, probably a deletion. Interference with synapsis seems the most likely explanation of the decreased frequency of donor phenotype transfer with *metB*-36 and, incidentally, the decreased frequency of transduction in the cross *metB-36* (×) wild-type in Table 4. In the former case this could have resulted from a reduction in size of the donor transducing fragments. If fragmentation of the donor chromosome in the preparation of phage lysates is not random (Ozeki, 1959), the formation of chromosomal fragments from *metB-36* carrying the *metB* and *F* loci should include a deletion and therefore be shorter.

Linkage relationships of the met *loci*

The linkage between two of the 5 *met* loci is a further example of linkage between loci concerned with related function in *Salmonella typhimurium* (Demerec, 1956*a*). Detection of linkage of other *met* loci may have been impossible because of fragmentation of the donor chromosome between them in the preparation of transducing phage (Ozeki, 1959). In some instances the linear linkage sequence of functionally related loci of *S. typhimurium* was the same as that of the sequence of metabolic blocks associated with each locus, i.e. an 'assembly line' was formed. Five of the 7 loci concerned with histidine (Hartman, Loper & Šerman, 1960) and all the 4 loci concerned with tryptophan biosynthesis (Demerec & Hartman, 1956) were shown to be arranged like this. Tryptophan biosynthesis in *Escherichia coli* involves the same pathway and is controlled by similarly linked loci (Yanofsky & Lennox, 1959). Examples of less perfect associations prevail amongst the cysteine (Clowes, 1958) and proline loci (Demerec *et al.* 1958) of *S. typhimurium*, where one pair out of 5 and one pair out of 4 loci, respectively, were linked and associated with consecutive metabolic steps. In any case, with the possible exception of the purineless mutants (Yura, 1956*a*; Demerec *et al.* 1956), whenever linkage between functionally related loci was demonstrated, the equivalent metabolic blocks appeared to be consecutive (Demerec *et al.* 1955, 1958). The sequence of metabolic blocks in the biosynthesis of methionine is *metA–metB–metC–*(*metE metF*) so that the linkage of the *metB* and *metF* loci constitutes an example of linkage between functionally-related loci not concerned with consecutive metabolic blocks.

The selective advantage of the assembly line of genes has been stressed both from the functional (Demerec & Demerec, 1955; Demerec & Hartman, 1956) and genetical (Hartman, Loper & Šerman, 1960) points of view. Linkage between *metB* and *F* indicates that at least two of the *met* loci are not arranged in an assembly-line sequence. However, within intact bacteria, folding and interfolding of the chromosome may ensure that functionally related loci are arranged in such a sequence (Pontecorvo, 1958). On the other hand, general acceptance of the genetical selective advantage of assembly lines to organisms with systems of partial genetic transfer should obviously be avoided.

Methionineless mutants of a number of different micro-organisms have been reported. However, appropriate biochemical and linkage data for comparison with the *met* mutants of *Salmonella typhimurium* are at present limited to strains of *Escherichia coli*, *Neurospora crassa* and *Streptomyces coelicolor*.

The information available on the methionineless mutants of *Escherichia coli* K12 suggests similarities with *Salmonella typhimurium* (Clowes & Rowley, 1954;

Ruebner, 1956; Dr F. Jacob, personal communication). On the basis of growth responses, three phenotypic groups are recognizable. They are equivalent to the *metA* + *metB* + *metC*, the *metE* and the *metF* mutants of *S. typhimurium*, respectively. In conjugation experiments one mutant of the first group (comprising 7 mutants) is linked to the only available representative of the last group (the M1 and M2 mutants of Clowes & Rowley, 1954). The other 6 mutants of the first group are closely linked together and located some distance from M1 and M2 on the circular linkage map of Pardee, Jacob & Monod (1959). Mutants of the second group are closely linked together but unlinked to any of the other methionineless mutants. It is interesting to note that the only linkage between phenotypically different methionineless mutants of *E. coli* involves mutants similar to those of the *metB* and *metF* loci of *S. typhimurium* which were the only *met* loci of this organism shown to be linked.

Seven mutants of *Neurospora crassa* associated with at least four different blocks in methionine biosynthesis have been recognized. Six of these have been mapped (Barratt, Newmeyer, Perkins & Garnjobst, 1954; Drs Catcheside & Murray, personal communication). None of these is closely linked although three are located on the same chromosome. Hopwood (1959) recognized 4 loci concerned with methionine biosynthesis in *Streptomyces coelicolor* but none of these showed close linkage to the others. Three linkage groups were suggested; 2 of the loci were situated in one and one each in the other two.

The following linkage map for some loci of *Salmonella typhimurium*, including the 5 *met* loci, was suggested by Demerec & Demerec (1955) and Glover (1955, 1956):

tryD–tryC–tryB–tryA–cysB–metE–cysA–metC–metA–metB–metF–(cysC cysD)

From the transduction studies reported in the present paper, the inter-locus donor phenotype selection data support the suggestion of linkage between *metB* and *metF* but not between *metC* and *metA* or *metA* and *metB*. Failure to detect donor phenotype transfer in transduction involving certain double auxotrophs as donors (Table 7) and the absence of prototrophs results from transduction between certain double auxotrophs and wild type (Table 8), provide no evidence for linkage between *tryA* and *metE*, *cysB* and *metE*, *metE* and *cysA*, *cysA* and *metC* and *metF* and *cysC*. These results and those of similar experiments by Clowes (1958, and unpublished) do not support the linkage map of Demerec and his colleagues, at least for the region to the right of *cysB*.

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Phenotypic Alterations Associated with the Bacteriophage Carrier State of *Shigella dysenteriae*

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SUMMARY

Shigella dysenteriae strain 136-R4 is lactose-negative, mannitol-negative (Lac^- $Mann^-$) and is sensitive to bacteriophage T7. Carrier clones of strain R4 (contaminated with T7) were found to maintain their association with phage T7 through as many as fifty consecutive single-colony isolations (from an ancestral colony which had survived lysis by phage T7). All carrier cultures (so-called pseudolysogenic strains) were found to be lactose-positive and mannitol-positive (Lac^+ $Mann^+$). Passage of Lac^+ $Mann^+$ bacteria through media containing antiserum directed against phage T7 resulted in a change back to Lac^- $Mann^-$ and in the complete elimination of phage T7.

Biochemical, genetic and immunochemical evidence indicates that the change from Lac^- $Mann^-$ to Lac^+ $Mann^+$ is the result of a phage-controlled alteration in the phenotype of *Shigella dysenteriae*. This dysentery bacillus is cryptic with respect to the expression of Lac^+ $Mann^+$ and the crypticity is attributable to surface structures (which are probably not a part of the Y or permease system). Under appropriate conditions decryptification may be brought about by phage-associated endolysin. Similar phenomena were observed in carrier strains of certain other members of the Enterobacteriaceae.

INTRODUCTION

In a previous communication (Barksdale, 1959) it was suggested that phage T7 is capable of converting Lac^- *Shigella dysenteriae* to Lac^+ *S. dysenteriae* and that this happens whether the phage T7 stocks have been derived from Lac^- or Lac^+ bacteria. In other words, an inherent property of bacteriophage T7 is responsible for the change from Lac^- to Lac^+. This phage conversion at first attracted our attention because we assumed *S. dysenteriae* to be genotypically Lac^- (Z^-) as had Luria & Burrous (1957*a*, *b*) and Cohn, Lennox & Spiegelman (1960). From the data to be presented here it is evident that some strains of *S. dysenteriae* are phenotypically Lac^- (cryptic) but genotypically Lac^+ (Z^+). Bacteriophage T7 produces an endolysin capable of decryptifying Shiga's bacillus (*S. dysenteriae*). This 'unmasking' is non-specific in that all strains which are Mal^- (maltose-negative), $Mann^-$ (mannitol-negative) and Lac^- become in the presence of phage (under suitable conditions) Mal^+ $Mann^+$ Lac^+. (Rough strains of *S. dysenteriae* grow feebly on maltose and on maltose MacConkey plates these organisms form pale pink colonies which are easily distinguishable from the red large colonies formed by phage-carrying clones.)

The association between phage and bacterium which makes possible these alterations in phenotype has been termed the carrier state or pseudolysogenic condition (Lwoff, 1953; Jacob & Wollman in Adams, 1959). The fact that the pseudolysogenic condition carries with it pseudogenetic effects is of some consequence for investigations concerned with bacterial and bacteriophage genetics. Decryptification by phage offers a useful tool for quickly determining the presence of hidden properties of bacteria not detectable in the phage-free state.

METHODS

Organisms. Aside from the strains of *Shigella dysenteriae* described herein, subcultures of the old rough strain Sh, originally used by Lisbonne & Carrère (1923) were kindly supplied by Dr G. Bertani (Sh Bertani) and by Dr S. Spiegelman (for E. S. Lennox) and by Dr S. E. Luria (Sh 15 and Sh 16). Strain Sh 60 was sent to us by Dr W. E. van Heyningen.

Escherichia coli strains of established genetic constitution with regard to the β-galactoside system were supplied by Dr J. Monod. We are indebted to Dr N. Zinder for his strain(s) of *Salmonella typhimurium*.

Media. MacConkey's agar base was compounded from (g.): Bacto-peptone, 17; Proteose-peptone, 3; NaCl, 5; Bacto-agar, 13·5; Bacto Neutral red, 0·025; water to 1 l., final pH 7·1. Sterilized by autoclaving at 120° for 20 min. Carbohydrate supplements were added as 20% (w/v) solution in distilled water, sterilized by filtration, sufficient being added (aseptically) to the molten agar base to yield 1% (w/v). Plates were poured to desired thickness.

Neopeptone broth consisted of Neopeptone Difco + 50% (v/v) fresh beef infusion. Neopeptone agar consisted of Neopeptone Agar Difco + 50% (v/v) fresh beef infusion.

PGT medium is the casein hydrolysate medium of Mueller & Miller (1941) as modified by Barksdale & Pappenheimer (1954).

Bacteriophage. All phages of the T series were derived from stocks maintained by the late Mark H. Adams. Phage PLT 22 *vir* (virulent) was kindly supplied by Dr N. Zinder (Rockefeller Institute). Methods and materials for phage assay were as described by Adams (1959).

Preparation of acetone powder of Shigella dysenteriae. Organisms from an actively growing culture were twice washed in saline in the cold (4°) and subsequently washed four times with eight volumes of cold acetone. Acetone from the final wash was decanted and the organisms dried to a white powder in vacuum. Plates for endolysin assay were prepared by adding 0·5 ml. of powder (resuspended in saline to optical density 5, at 590 mμ as measured in a Bausch & Lomb Jr. spectrophotometer) to 2 ml. molten soft agar and plating as a soft agar layer according to the method described by Adams (1959).

Assay for β-galactosidase. For the assay of this enzyme the method of Pardee, Jacob & Monod (1959) was modified as follows. Toluenized extracts were not prepared because, except for organisms grown in maltose, toluenized extracts of the shigella strains used here proved to have less activity than unextracted organisms. Instead, organisms were mixed with 10^{-3}M-*o*-nitrophenyl-β-D-galactoside in 0·25 M-sodium phosphate buffer and incubated for 10 min. at 28°. The reaction was then halted by the addition of 4 ml. cold M-Na_2CO_3 and the organisms removed by centrifugation in the cold. The supernatant fluid was decanted and its optical density read in the Zeiss spectrophotometer at 420 mμ. The unit of enzyme was that amount which yielded 1 mμmole-*o*-nitrophenol/min. at 28°.

RESULTS

Shigella dysenteriae wild type (strain Sh 136-T) was isolated by one of us (L.B.) from a case of Shiga dysentery in Tokyo in 1947. Strain 136-T forms smooth translucent colonies on Neopeptone agar; it possesses a characteristic surface antigen and exhibits a low but definite virulence for mice (Barksdale, Okabe & Li, unpublished). It is resistant to the phages T3, T4, T5 and T7. It does not ferment lactose, mannitol or maltose. A commonly encountered mutant of strain Sh 136-T forms opaque colonies (strain Sh 136-0) on Neopeptone agar, is antigenically distinguishable from wild-type, is of a lower order of virulence for mice and can support the multiplication of phage T7. From strain Sh 136-0 a number of rough mutants have been derived. Those important to this report are Sh 136-R1, Sh 136-R2 and Sh 136-R4. Those are all less virulent for mice than strains 136-T and 136-0. They belong to the class of rough *Shigella dysenteriae* and therefore have much in common with the rough strains: Sh (Bertani), Sh 15 of Luria (see Adams & Luria, 1958) and Sh 60 of Van Heyningen (1955). It can be seen from Table 1 that rough strains were capable of adsorbing and supporting the growth of more different phages of the T series than was the wild-type 136-T. For example, strain 136-T is resistant to phage T7, whereas strain 136-R4 is sensitive to T7.

Table 1. *Efficiency of plating* of coliphages on* Shigella dysenteriae

	Bacteriophage							
	T1	T2	T3	T4	T5	T6	T7	$T7^{Sb}$
	Efficiency of plating (%)*							
Strain								
Sh 136-T	84	1	r	r	r″	5	r	0·3
Sh 136-0	180	39	r	r	r″	0·01	1·8	1·0
Sh 136-R1	100	85	97	r	46	96	195	150
Sh 136-R2	127	93	50	r	81	22	110	350
Sh 136-R4	103	86	93	r″	71	81	432	520
Sh (60)	r	73	95	0·001	r	1	126	33
Sh (Bertani)	102	0·01	14	100	180	100	104	67
Sh 15	93	90	0·5	26	67	54	98	130

$$* \text{ EOP} = \frac{\text{Number of plaques formed on Shigella}}{\text{Number of plaques formed on } E.\ coli \text{ B}} \times 100.$$

r = resistant, as indicated by failure of plaque formation in any dilution of stocks containing 10^{10} particles/ml.

r″ = resistant but lysed by phage endolysin (this category includes lysis from without).

Though sensitive to phage T7, clones of 136-R4 were found to enter a carrier relationship with this bacteriophage, so that the virus persisted with the bacteria through many generations. Our carrier strains were first isolated as supposed bar mutants of 136-R4 resistant to phage T7 (136-R4/7). Drops (approximately 0·025 ml.) of phage T7 (5×10^{10} particles/ml.) were placed on lawns of 136-R4.

After several days of incubation at 30° there appeared in the middle of the lysed area, colonies of resistant bacteria (see Demerec & Fano, 1945). Some of these were picked and streaked on agar plates to obtain isolated clones free from contaminating phage. A number of the colonies derived from this first plating exhibited a nibbled appearance. Restreaking of entire (non-nibbled) colonies yielded some nibbled and some entire colonies. After fifty successive restreakings of entire colonies, nibbled colonies and entire colonies continued to segregate. Since T7 is a virulent phage, it seemed unlikely that it had been perpetuated as prophage in the cultures. Cultivation of phage-carrying clones in anti-T7 serum resulted in a complete elimination of the virus. The phage-free bacteria so treated were again sensitive to T7.

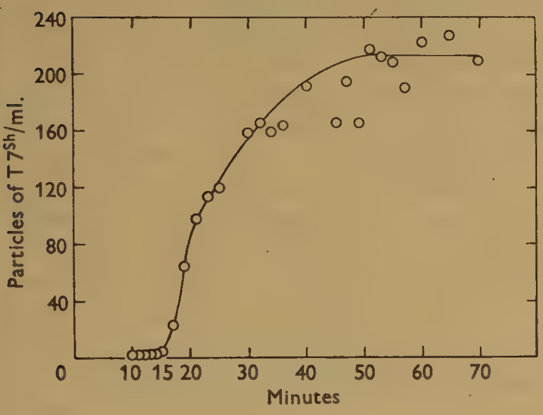
Presence of phage associated with Lac$^+$ condition

When the streaking which led to the segregation of entire and nibbled colonies was carried out on lactose agar plates containing neutral red as an indicator (MacConkey's agar), red colonies (lactose-positive) and colourless colonies (lactose-negative) developed on the plates. Some of the red colonies were obviously nibbled and some were entire. When 10 red colonies were suspended in 1 ml. broth, centrifuged and the supernatant fluid assayed for phage, phage was present in each of the 10; none of 10 colourless (Lac$^-$) colonies, so examined, contained phage. When the bacteria from a Lac$^+$ colony were suspended in anti-T7 serum and subsequently plated on lactose plates, only Lac$^+$ colonies grew out. Thus it appeared that Lac$^+$ colonies always carried virus and, on subculture, always segregated Lac$^+$ and Lac$^-$ colonies. Furthermore it became apparent that Lac$^+$ was a more reliable index to the presence of virus in a colony than was the state of the margin of the colony (nibbled *versus* entire). The first question these observations raised was 'where is the lactose-positiveness coming from?' The phage T7 preparations were originally grown on *Escherichia coli* B, a lactose-positive organism. So, starting from a single plaque of T7 on 136-R4 (a supposed lactose-negative organism) a stock of T7 was prepared by growth on this shigella. The preparation of phage so obtained was designated T7$^{\text{Sh}}$. It differed from T7 slightly (see Table 2) but was able satisfactorily to establish the carrier condition with 136-R4. The carrier clones were Lac$^+$.

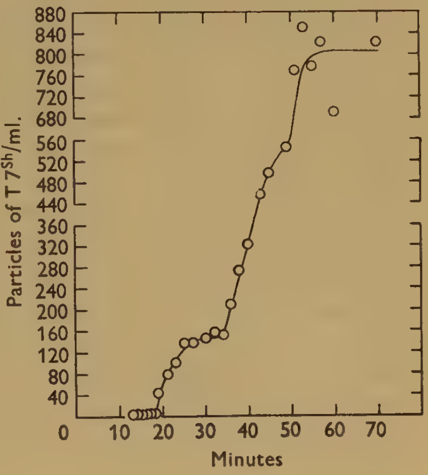
Table 2. *Host ranges of phages T7 and T7$^{\text{Sh}}$*

Bacterium	Lysis by T7	Lysis by T7$^{\text{Sh}}$
Shigella 136-T	−	+
Shigella 136-R4	+	+
Coli W-2241	+	−

One interesting fact about the phages T7 and T7$^{\text{Sh}}$ multiplying on Shigella 136-R4 is worthy of note. The one-step growth curves obtained from the growth of T7 and T7$^{\text{Sh}}$ on strain *Escherichia coli* B were quite different from those obtained on shigella 136-R4. In the case of strain 136-R4 there appeared to be two separate bursts: one occurred from the 19th to the 30th minute; the other, from the 35th to the 52nd minute. The greatest increment of phage came from the late-lysing population. This effect was observed only under standard conditions of phage growth (Adams, 1959) where nutrient broth or Neopeptone broth were employed. Experiments carried out in the semi-defined PGT medium (casein hydrolysate) yielded data similar to that shown in Fig. 1*a*.



a



b

Fig. 1*a*. One-step growth curve of T7^{Sh} grown on *Escherichia coli* B. Multiplicity of phage to bacterium = 0·1. Multiplicity was calculated from titre as determined on *E. coli* B (concentration of bacteria: $1{\cdot}5 \times 10^8$ bacteria/ml.).

Fig. 1*b*. One-step growth curve of T7^{Sh} on *Shigella dysenteriae* 136-R4. Multiplicity of phage to bacterium = 0·1. Multiplicity was calculated from titre as determined on Sh. 136-R4 (concentration of bacteria $1{\cdot}5 \times 10^8$ bacteria/ml.).

The carrier colony

Carrier colonies which grew out on MacConkey's agar developed as completely red (lactose-positive) colonies or as sectored colonies (lactose-positive sectors alternating with lactose-negative sectors). As the sectored colonies aged, they became completely red. Restreakings of sectored colonies yielded Lac$^+$ and Lac$^-$ colonies. Microscopic examination of organisms from Lac$^-$ colonies revealed short rod-shaped bacteria. Organisms from Lac$^+$ colonies were swollen spheroplast-like structures; sometimes with pieces of material adhering to their surfaces, much as portions of cell wall adhere to the membranes of freshly prepared spheroplasts (See drawings in Lederberg, 1956, which show a similar appearance). Occasionally, these swollen structures occurred in short chains of three to five units flattened at their abutting sides. Larger fragments of cell wall appeared to be retained by organisms in chains than by single units. These spheroplast-like structures (demi-spheroplasts) were osmotically fragile. They were not observed on Gram-stained preparations but were readily visible in wet mounts prepared in 20% (w/v) sucrose. When the bacterial mass was taken from one colony, suspended in sucrose broth, diluted in sucrose broth and plated, the number of viable units was about 5×10^5. This is one hundred to one thousand times fewer viable units than one would expect from an uninfected bacterial colony of similar size.

Osmotic fragility of carrier clones

The osmotic fragility of a carrier colony was tested by suspending the colony in sucrose, diluting half of the suspension in sucrose broth and half in plain broth and comparing the viable units obtained by plating on MacConkey's agar. Eighty % more survivors occurred on the platings from sucrose than on those from broth. Seventy-five % of the sucrose survivors were Lac$^+$ whereas only 12 % of the broth survivors were Lac$^+$, thus suggesting that the organisms likely to give rise to Lac$^+$ clones were osmotically more fragile than those likely to give rise to Lac$^-$ clones. A probable explanation for the extreme fragility of these organisms was found. Phages T7^{8h} and T7, like so many bacteriophages, produce plaques with a central clear area surrounded by a halo (opaque zone) in which the appearance of the bacterial mass is different from that existing beyond the plaque and comprising the unaffected lawn. While the bacteria in the halo have an obvious alteration in their optical properties, they are not infected with phage; no phage can be obtained from them. The endolysin responsible for their appearance is also capable of bringing about the lysis of dead bacteria. In Pl. 1, figs. 1, 2, is shown a hole formed in a lawn of dead dysentery bacilli (acetone-powder) by phage T7 endolysin.

It is very well established that endolysins are capable of removing specific phage receptors (Adams & Park, 1956; Adams, 1959). Bacteria which have lost their receptors, either by enzymic action or as a result of mutation, are immune to attachment by the whole phage (Spizizen, 1957). Thus the bacteria in a halo are immune to phage much as are spheroplasts. The bacteria in a *Shigella dysenteriae* carrier strain presumably owe their immunity to endolysin generated by the small number of bacteria in which phage succeeds in multiplying.

How does the first bacterium of a carrier clone survive infection? In Table 3 is shown the incidence of Lac$^+$ survivors at various multiplicities of phage to bacteria. Each of these Lac$^+$ clones must have been initiated by a bacterium which had escaped lysis. Fraser (1957) in experiments carried out in microdrops established that certain mutants of phage T3 gave rise to lasting complexes with *Escherichia coli* B. 'The complexes' (bacteria infected with phage) 'can increase in length, divide and give phage-sensitive uninfected segregants before lysis'. We found that phage T3 gave Lac$^+$ carrier clones with both *Shigella dysenteriae* and *S. sonnei*. Whilst we have carried out no single-cell studies, we feel that Fraser's findings offer a reasonable explanation for the origin of our carrier clones: the initially infected ancestral bacterium fails to lyse, reaches a spot on an agar plate, divides to give rise to an infected and uninfected daughter. This process may be repeated several times before lysis actually occurs. (Of course a few bacteria in the population might very well have their receptors destroyed and escape infection and arrive on the plating medium in a receptorless state, etc.). With lysis there is liberation of phage and endolysin. Bacteria geographically so located that they absorb phage may subsequently lyse. Bacteria bathed in endolysin become immune as they lose their receptors. Eventually, an equilibrium is reached between phage, susceptible bacteria and immune bacteria. Susceptible bacteria are necessary for continued generation of endolysin. This equilibrium may be destroyed by antiphage serum or by dilution into broth or other media having little or no osmotic buffering capacity.

Table 3. *Incidence of carrier clones* (136-*R*4.$T7^{Sh}$) *when* 1×10^8 *organisms of* Shigella dysenteriae 136-*R*4 *were infected with various multiplicities of phage* $T7^{Sh}$

Ratio of phage to bacteria	Dilution of infected culture which was plated	No. of Lac^+ survivors	Lac^+ survivors as % of total survivors
50	10^{-1}	0	—
	10^{-2}	2	33
	10^{-3}	27	28
	10^{-4}	2	67
10	10^{-1}	0	—
	10^{-2}	8	12
	10^{-3}	20	33
	10^{-4}	2	40
1·0	10^{-1}	0	—
	10^{-2}	30	23
	10^{-3}	3	60
	10^{-4}	0	—
0·1	10^{-1}	0	—
	10^{-2}	10	5
	10^{-3}	1	2
	10^{-4}	0	—

Evidence for the presence of β-galactosidase in Shigella dysenteriae

*The effect of methyl-thio-β-*D*-galactoside* (*TMG*) *on the degree of β-galactosidase activity in culture of* 136-*R*4. Following a discussion with Dr H. Rickenberg about the possible presence of β-galactosidase in *Shigella dysenteriae*, we found that 136-R4 organisms grown in PGT medium produced appreciable amounts of enzyme when methylthio-β-D-galactoside (TMG) was present. In Table 4 are shown the results of growing strain 136-R4 in PGT medium with no added carbohydrate, with 2% maltose and with 10^{-3}M-TMG.

Table 4. *β-Galactosidase synthesis by* Shigella dysenteriae 136-*R*4 *Grown in PGT medium*

	+ Maltose	+ TMG	No addition
Optical density*	3·1	2·6	2·75
Units of enzyme	0·11	11·6	0·026
$\frac{\text{Units enzyme}}{10^9\text{ bacilli}}$	0·035	4·4	0·009

* O.D. 1·0 = 1×10^9 136-R4 organisms/ml.

Similar results were obtained with Shigella strain Sh 60. Strain Sh 15, however, showed about $\frac{1}{200}$th the activity of shigella 136-R4.

The occurrence of Lac^+ *mutants of* 136-*R*4. When bacteria were plated so that about 5 to 10 well-isolated colonies developed on a single plate the diameter attained by each colony (with prolonged incubation) might be as much as 10–15 mm. (so-called giant colonies). Thick MacConkey's agar plates (lactose) containing giant colonies of strain 136-R4 were sealed with masking tape and incubated for 8 weeks at 30°. During this time several red papillae appeared on the colonies of 136-R4. Four bacterial lines developed from an equal number of papillae (136-R4-L-1, -L-2, -L-3 and -L-4) all proved to give positive fermentation reactions in standard lactose fermentation tubes. When one of these mutants was tested for enzyme activity, its basal level was found to be much greater than that of 136-R4 (see Table 5).

Table 5. *β-Galactosidase production by mutant shigella* 136-*R*4-*L*-1 *when grown in PGT medium with the indicated additions*

	No addition	Maltose 2%	Glycerol 2%	Glucose 2%	Lactose 1%	TMG* 10^{-3} M	IPGT† 10^{-4} M
Optical density	2·8	3·3	2·2	1·4	2·8	1·8	2·8
$\frac{\text{Units } \beta\text{-galactosidase‡}}{10^9 \text{ bacilli}}$	5·8	6·3	3·3	2·9	15·1	5·3	11·2

* The presence of TMG in the medium resulted in a 'stickiness' of the bacteria, i.e. on standing they sedimented to form a tightly adhesive mass. This phenomenon was observed only in the case of organisms grown with TMG.

† Isopropyl-thio-*β*-D-galactoside was kindly provided by Dr S. E. Luria from a sample from Dr D. Türk (Institut Pasteur, Paris).

‡ 1 unit enzyme is that amount which produces 1 mμmole *o*-nitrophenol/min. at 28°, pH 7·0 (see Pardee *et al.* 1960).

The occurrence of Lac$^+$ recombinants in the cross Hfr (Z^-) × 136-*R*4. It was previously established (Barksdale, 1959) by radioisotope experiments carried out with Dr M. J. Osborn of this department that, under conditions where *Escherichia coli* ML30 showed normal uptake of labelled ^{35}S thio-*β*-D-digalactoside, 136-R4 was unable to concentrate this substrate. If 136-R4 is truly a cryptic strain, then one should be able to eliminate its crypticity by bringing about a genetic alteration of its surface. Mating between shigella and *E. coli* strains was first accomplished by Luria & Burrous (1957*b*). We have for some time been using this ability to mate as one of several means of characterizing various strains of *Shigella dysenteriae* and we find that rough strains undergo mating and recombination with various Hfr strains of *E. coli*. Dr S. E. Luria kindly supplied us with a strain of Hfr characterized as: Hfr (λ) M^-Pr^- Lac 72 (extreme Z^- type deletion). The genetic designations Y (permease), Z (*β*-galactosidase) and i (inducibility) refer to that *Escherichia coli* chromosomal segment governing the *β*-galactoside system. By using a streptomycin-resistant mutant of strain 136-R4 (136-R4 S^r) as an F^- (acceptor) parent, a mating was carried out: 1×10^8 organisms of 136-R4 S^r Lac$^-$ *non-motile* were combined with 3×10^9 organisms of *Hfr* S^s *Lac*$^-$ (*Z deletion*) *motile* and incubated with gentle shaking for 100 min. Dilutions of the mating mixture were then plated on MacConkey's lactose agar containing 1 mg. streptomycin/ml. Control platings were made of the donor strain and of the acceptor strain. After incubation for 48 hr. at 37° Lac$^+$ colonies were found on platings from the lower dilutions of the mated mixture. There were no Lac$^+$ colonies on the acceptor control plates; there was no growth on the donor control plates. Following restreaking and 'purification', the recombination clones proved to be non-motile, S^r and frankly lactose-positive organisms. Since both parental organisms were Lac$^-$ and since it is well established that the donor strain harbours a deletion with regard to Z^+, it seems evident that Z^+

of the recombinants must have been furnished by the shigella parent. The Lac^+ condition (arising from the cross of $Lac^- \times Lac^-$) in all probability resulted from the influence of donor genes upon the surface properties (permeability to β-galactosides) of the recombinant. In this connexion it should be pointed out that some years ago Luria & Burrous (1957*a*) found a correlation between alteration in serotype and lactose-positiveness in crosses between *E. coli* and *Shigella flexneri*. They were so impressed with this apparent relationship (between serological type and Lac^+) that they stated: 'The new serotype is controlled by a genetic factor closely linked to, but not identical with, the Lac locus.'

The relationship of Escherichia coli *β-galactosidase to the β-galactosidase of* Shigella dysenteriae

We are much indebted to Drs M. Cohn, S. Spiegelman and E. Lennox for a generous supply of purified *Escherichia coli* β-galactosidase and of its antiserum (rabbit). Without these we should not have attempted to establish the immunochemical identity of the β-galactosidase derived from *E. coli* and that derived from *Shigella dysenteriae* strain 136-R4. The simplest method of making an immunochemical survey of the various enzymes, toxins and other antigens produced by microorganisms is through the use of crude lysates and purified known antigens and antibodies reacting in an agar medium. (It was while experimenting with phage T7 endolysin as an agent for releasing Shiga neurotoxin that we observed the Lac^+ carrier clones of *Shigella dysenteriae*.) In Pl. 2, fig. 4 is shown the reaction of β-galactosidase (released by a $T7^{Sh}$.136-R4 carrier clone) from lysing *S. dysenteriae* with anti-serum against *E. coli* β-galactosidase. It can be seen that the lines formed by the reaction shigella β-galactosidase *v.* coli anti-β-galactosidase are continuous with the lines resulting from the reaction of purified coli β-galactosidase *v.* coli anti-β-galactosidase. The plate shown in Pl. 2, fig. 4 was set up in the following way. Plates of MacConkey's agar base (without neutral red) containing 10^{-3}M-TMG as the only added 'carbohydrate' were poured to yield thick layers of agar. Upon each plate were placed 3 disks of Whatman no. 50 filter paper (diam. 13 mm.) as shown in Pl. 2, fig. 4. To disks 1 and 3 were added 0·03 ml. undiluted rabbit antiserum directed against a purified β-galactosidase. To disk number 2 was added 0·02 ml. containing 360 μg. (protein) of purified β-galactosidase. To position 4 was added 0·01 ml. of a freshly prepared lysate of strain 136-R4 resulting from the action of bacteriophage $T7^{Sh}$ (5×10^9 T7 particles/ml. in combination with 1×10^9 136-R4 organisms/ml.). After incubation for 7 days at 30° definite single lines developed between the β-galactosidase (position 2) and the anti-β-galactosidase (positions 1 and 3) and these fused with the major component which developed between the carrier clone and the anti-β-galactosidase (of positions 1 and 3). There seems little doubt, then, that the β-galactosidase of *E. coli* and *S. dysenteriae* 136-R4 are immunochemically identical. Similar results obtained with strains Sh 60 and Sh 15. The latter strain produce very small amounts of enzyme.

Non-specific nature of the effect of bacteriophage on the phenotype of Lac^- strains

The key character upon which depends the taxonomic delineation of subgroups in the genus *Shigella* is that of mannitol fermentation (+ or −). *S. dysenteriae* is mannitol-negative. It seemed evident to us that if the effect of phage T7 on the phenotype 136-R4 was merely an alteration of permeability by endolysin action, then permeability to some other sugars not used by *S. dysenteriae* should also be altered in clones of 136-R4 carrying phage T7^{Sh}. This would be true were *S. dysenteriae* cryptic with respect to enzymes needed for the dissimilation of certain other sugars. MacConkey agar plates containing salicin, mannitol or maltose were prepared and Lac$^+$ carrier clones from lactose plates (MacConkey agar) were transferred to these. All Lac$^+$ clones proved to be maltose-positive and mannitol-positive but not salicin-positive. As indicated earlier, on maltose plates *S. dysenteriae* formed slightly pink colonies; the carrier clones yielded bright red colonies which attained a larger size than uninfected colonies.

The universality of the effect of bacteriophage on the phenotype of carrier clones

When the Zinder strain of *Salmonella typhimurium* (stm) becomes infected with a virulent mutant of phage PLT 22 (Zinder, 1957) the carrier state is established. These carrier clones show no alteration in their fermentative properties on lactose plates. However, on maltose and on mannitol agar the infected populations formed deep red colonies in contrast to the pink ones formed by uninfected populations. Passage of infected populations through anti-PLT 22 serum (kindly supplied by Dr Zinder) restored the native state of *S. typhimurium*.

Escherichia coli K12 W2241 ($Y^-Z^+i^+Mal^+$) when infected with phage T7^{Sh} at a multiplicity of 5 yielded 2·1 Lac$^+$ survivors/10^7 bacteria. Among Lac$^+$ colonies developing on MacConkey medium plates some were carriers and some contained Lac$^+$ back mutants. Plating of the same material on mannitol plates, however, clearly distinguished between red carriers and colourless uninfected survivors. Neutralization of red clones with T7 antiserum resulted in a return to the 'colourless' state.

In the case of the formation of carrier colonies between phage T7^{Sh} and *Escherichia coli* K12 W2242 ($Y^+Z^-i^+Mal^+$) and *E. coli* K12 W2244 ($Y^+Z^-i^+Mal^+$), no Lac$^+$ clones were detected. Red phage-infected colonies did develop on mannitol plates and on maltose plates, however, and this condition was reversed by passage in anti-T7 serum. Carrier clones of Hfr Z^- (W-4032) were also lactose-negative. *E. coli* ML3 ($Y^-Z^+i^+$) and ML3080 ($Y^-Z^-i^-$) were both resistant to phages T7 and T7^{Sh}. When combined with either of these phages and plated on lactose plates, no Lac$^+$ clones developed. The high rate at which these strains mutated to Lac$^+$ made it necessary to select a fresh Lac$^-$ colony for each experiment.

DISCUSSION

It is a common practice among geneticists to characterize mutant organisms over the common denominator of their ancestral wild type. There is always some variation among wild strains and the taxonomists in selecting type strains (ideally) attempt to choose one that is a happy medium of those extremes found in nature. Some years ago we examined several wild type strains of *Shigella dysenteriae* in an effort to find out what, if anything, distinguishes this organism, freshly isolated from cases of dysentery, from various laboratory strains designated as *S. dysenteriae*. Wild type *S. dysenteriae* is a smooth organism with specific immunochemical, biochemical and pharmacological properties. From several wild types isolated from cases of Shiga dysentery, we have selected strain 136T as a typical Shiga bacillus (and maintained it as lyophilized 'copies' all 'fresh from nature'). The series of stepwise mutations presented here, from 136-T Lac⁻ to 136-0 Lac⁻ to 136-R1 Lac⁻ to 136-R4 Lac⁻ are probably the same steps by which strains Sh15, Sh60 and other rough strains, considered in this paper, came into being. While the wild type is resistant to several of the coliphages, various colony-form mutants, which exhibit different surface antigens, are sensitive to a wide range of coliphages. Mutant rough strains, several steps removed from the wild type, are sensitive to all of the standard coliphages. It is from this latter group of extreme-rough organisms that one isolates Lac⁺ *S. dysenteriae*. Considered as a mutant of the wild type, a Lac⁺ mutant represents then a very rare event.

Our evidence seems to indicate that *Shigella dysenteriae* is cryptic with respect to β-galactosidase (and to the enzymes involved in maltose and mannitol utilization). This 'walled off' state is attributable to surface properties peculiar to Shiga's bacillus and has, probably, only an apparent relation to β-galactoside system. The lactose utilization system in *Escherichia coli* involves three effects which, genetically, are closely linked: β-galactoside permease (Y), β-galactosidase (Z) and the inducible condition (i) (see Pardee *et al.* 1959). While the β-galactosidase of *S. dysenteriae* is immunochemically identical with that of *E. coli*, there is as yet no evidence that the two organisms harbour identical transport (permease) systems. If they do, then other surface structures in the case of Shiga's bacillus serve to block the permease (Y) thus rendering the organism phenotypically Y^-. Such a masking of phenotype would be analogous to the state of the mucoid mutant of a phage-sensitive bacterium which is itself resistant to phage, not because it lacks phage receptors but because its phage receptors are covered by a surface layer of polysaccharide.

It is not surprising that *Shigella dysenteriae* has an enzyme in common with *Escherichia coli*, for interfertile populations usually have much in common. As a matter of fact the number of cross-reacting antigens shared by *S. dysenteriae* and *E. coli* B, for example, appears to be considerable, for when lysates of these organisms are placed on areas of agar equidistant from Shiga antitoxin, several common antigen-antibody lines are formed. These lysates (complexes of phage and bacterium) offer a simple and effective means of releasing for immunochemical identification various biologically-active macromolecules not ordinarily detected in micro-organisms or as their extracellular products. It is possible by removing borings of agar (with a capillary pipette) at various distances from the phage + bacterium complex on TMG plates, to obtain samples having different concentrations of β-galactosidase. By this means β-galactosidase activity can be detected in strains producing only slight amounts of active enzyme. In examining carrier clones of the Z^- coli strains furnished by Dr J. Monod we observed one strain which produced an impressive β-galactosidase anti-β-galactosidase line in TMG MacConkey agar but which showed only an extremely small amount of β-galactosidase activity. Presumably, most of the β-galactosidase produced by this strain was inactive (see Perrin *et al.* 1959). We mention this here because it has some bearing on the quality and amounts of β-galactosidase to be expected in other micro-organisms.

Shigella dysenteriae strain Sh15. By conventional methods of examination the Sh15 strain of *Shigella dysenteriae* would appear to be Z^-. We were unable to obtain Lac⁺ mutants of it under the conditions here described for selecting Lac⁺ mutants of 136-R4. It is possible, however, to prepare Lac$^+$ complexes between strain Sh15 and phage T7^{8h}. The fusion of faint but definite antigen-antibody lines, formed between these complexes (T7^{8h}, 136-R4) and anti-β-galactosidase (rabbit serum), with bona fide β-galactosidase anti-β-galactosidase lines has been observed. This indicates that the Z-region is present in strain Sh 15. Dr S. Luria (personal communication) has additional evidence which suggests that strain Sh15 is Z$^+$. We pointed out (Table 5) that strain Sh15 produced minute amounts of active enzyme. In addition, its output of material immunochemically reactive with anti-β-galactosidase was low. It would seem, therefore, that though strain Sh15 is Z$^+$, the Z-region of its genome is much impaired.

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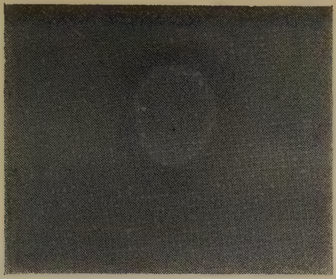

Fig. 1

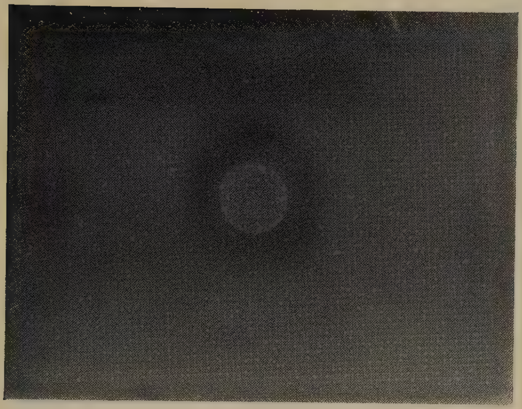

Fig. 2

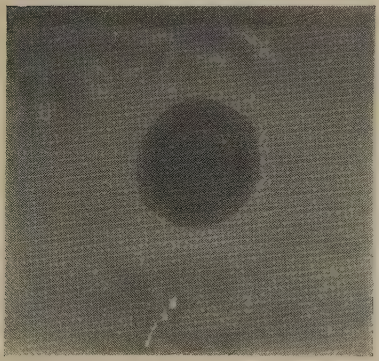

Fig. 3

KOIBONG LI, L. BARKSDALE AND L. GARMISE (*Facing p.* 366)

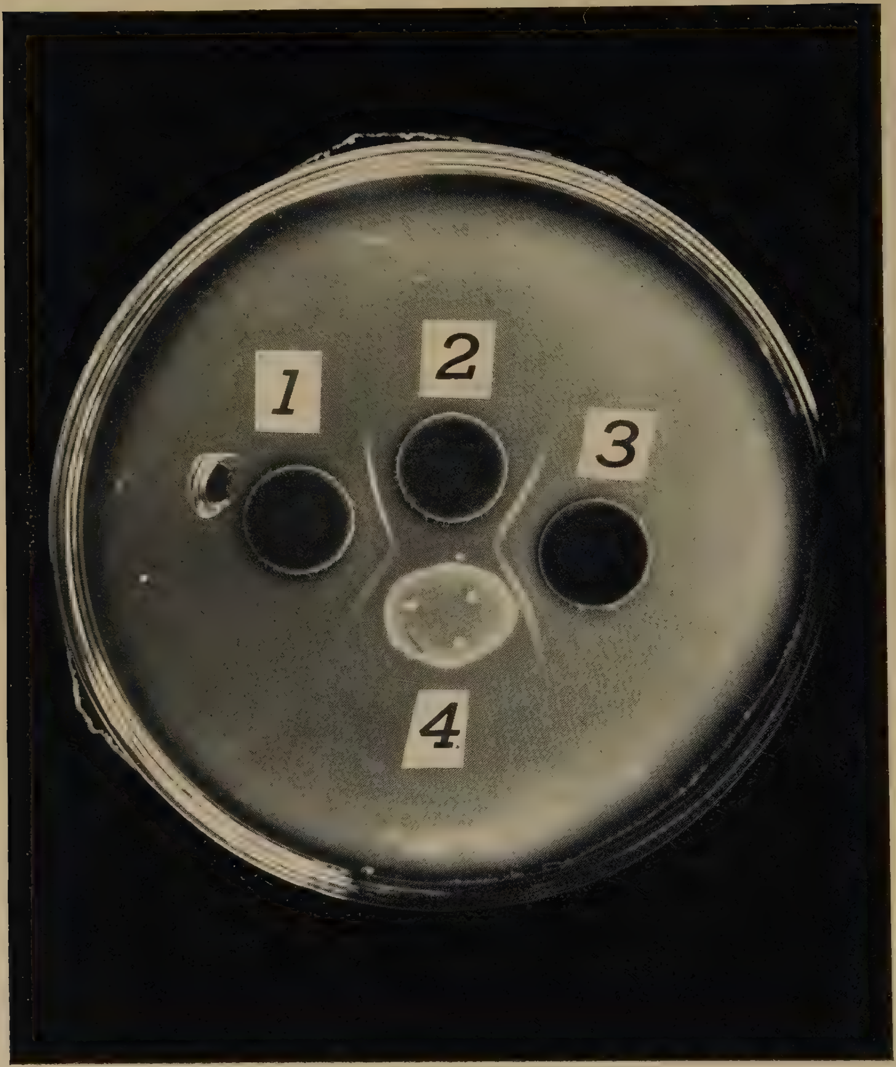

Fig. 4

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EXPLANATION OF PLATES

Plate 1

Figs. 1 and 2 are photographs of holes produced in a lawn (prepared from an acetone powder of *Shigella dysenteriae* 136-R4) by a carrier clone (lysate) of 136-R4.T7^{Sh}.

Fig. 3. A photograph of the lysis of living 136-R4 caused by the diffusion of phage and endolysin from carrier clone (lysate) of 136-R4.T7^{Sh}. Note the differentiation of the lysed area into clear inner portion and outer halo.

Plate 2

Fig. 4. The fusion of antigen-antibody lines formed between the β-galactosidase released by carrier clone 136-R4 (Lac$^-$).T7^{Sh} (position 4) with antigen-antibody lines formed between purified β-galactosidase (position 2) and rabbit antiserum directed against β-galactosidase (positions 1 and 3).

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Stimulation of Streptomycin-Resistant Bacteria in the Rhizosphere of Leguminous Plants

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SUMMARY

Bacteria which were resistant to streptomycin and rose bengal were preferentially stimulated in the rhizospheres of several plant species as compared with those in the soil away from the rhizosphere. Leguminous plants were the most effective and clover plants as young as 6 days had some effect. The separate and combined effects of rose bengal and streptomycin in the isolation medium were examined. Rose bengal alone had little effect on bacterial numbers, whereas streptomycin alone decreased them. The two substances together affected rhizosphere numbers strikingly but inconsistently. The bacteria from rhizosphere and soil were grouped according to their nutritional requirements. Chromogenic bacteria with simple requirements were the most abundant forms resistant to streptomycin + rose bengal in the rhizosphere and a species of *Flavobacterium* was especially favoured.

INTRODUCTION

Media for the isolation of soil fungi usually have antibacterial agents added, otherwise bacterial growth spreads over the fungal colonies. Several such agents have been used. Martin (1950) found that a mixture of streptomycin + rose bengal at 30 and 15 mg./l., respectively, was very effective. Rose bengal restricts fungal colony size and is also bacteriostatic; streptomycin inhibits only the bacteria. Martin found that the few bacteria which did develop in media containing these substances did not interfere with or decrease fungal counts. Bakerspigel & Miller (1953) used streptomycin in media for the isolation of *Heterosporium iridis* and found that it inhibited equally bacteria from soil and from the rhizospheres of iris, potato and bean. In contrast Peterson (1957), with media containing streptomycin + rose bengal found enough bacteria on plates prepared from the rhizosphere of clover plants to interfere with fungal isolations, but not on plates prepared from the corresponding non-rhizosphere soil. There was thus a preferential stimulation of the resistant bacteria in the rhizosphere; sugar beet and wheat had a similar effect but less so than the leguminous species. Such preferential stimulation does not seem to have been recorded previously and further investigation appeared worth while.

METHODS

Rothamsted clay loam soil (Pastures field; pH 6·6) was used. The plants were grown in a greenhouse in 4 in. glass pots of soil, watered as required; they were not watered during 24 hr. before sampling. Rhizosphere samples were obtained by carefully lifting the plants and shaking them to remove most of the soil adhering to the roots, which were then cut off at the crown and dropped into a screw-capped bottle containing 100 ml. sterile distilled water; enough roots were used to provide about 1 g. rhizosphere soil. The bottle was then shaken on a machine for 10 min. at 150 oscillations/min. with a horizontal excursion of 1 in. Serial ten-fold dilutions were prepared and three replicate plates were made from suitable dilutions. After removing roots the contents of the bottle and the subsequent dilution were evaporated to dryness to find the dry weight of soil. The numbers of bacteria were finally calculated per g. dried soil adhering to the roots.

Non-rhizosphere samples were taken from unseeded pots of soil which had been treated similarly to the seeded ones. The surface quarter inch of soil was removed with a sterile spatula and about 1 g. soil taken from the upper two inches and put in a bottle containing 100 ml. sterile distilled water. From this point the procedure was like that described for the rhizosphere samples.

Media for making colony counts. The numbers of bacteria in rhizosphere and non-rhizosphere soils were estimated from colony counts after incubation for 7 days at 25° on glucose peptone agar (glucose, 10·0 g.; peptone, 5·0 g.; KH_2PO_4, 1·0 g.; $MgSO_4.7H_2O$, 0·5 g.; agar, Difco, 20·0 g.; tap water to 1 l.; pH 7·0). The numbers of bacteria resistant to streptomycin + rose bengal were estimated from colony counts made on the above medium to which streptomycin to 30 mg./l. was added just before pouring the plates and rose bengal to 33 mg./l. added during the preparation of the medium (GPStRb) agar. Bacterial numbers were also estimated from colony counts after incubation for 7 days at 25° on soil-extract agar (K_2HPO_4, 0·2 g.; agar, Difco, 20·0 g.; soil-extract, 1000 ml., prepared by autoclaving 1 kg. soil with 1 l. distilled water for 30 min., filtering after adding a little $CaSO_4$, and making filtrate up to 1 l.; pH 7·0).

Nutritional grouping. The bacteria from the rhizospheres and soil samples were grouped according to the method of Lochhead & Chase (1943) by testing whether they would grow on the following media:

(*a*) Basal medium: glucose, 1·0 g.; K_2HPO_4, 1·0 g.; KNO_3, 0·5 g.; $MgSO_4.7H_2O$, 0·2 g.; $CaCl_2$, 0·1 g.; NaCl, 0·1 g.; $FeCl_3$, 0·01 g.; distilled water to 1 l. Heat to 100°, filter, and adjust to pH 6·8.

(*b*) Basal medium + amino acids: basal medium + 0·05 g./l. each of cysteine, alanine, proline, asparagine, glutamic acid, aspartic acid, arginine, leucine, glycine and lysine; pH 6·8.

(*c*) Basal medium + yeast extract: basal medium + yeast extract (Difco), 1·0 g./l.; pH 6·8.

(*d*) Basal medium + yeast extract + soil extract: 750 ml. basal medium + 250 ml. soil extract (prepared as above) + yeast extract 1·0 g./l.; pH 6·8.

RESULTS

Experiments were made to test for rhizosphere stimulation of bacteria resistant to streptomycin + rose bengal with a wide range of plant species. The plants were all grown in Pastures field (Rothamsted) soil and sampled first when they were well developed and at intervals afterwards. Table 1 expresses the results in terms of the ratio number of bacteria in rhizosphere to number of bacteria in corresponding soil

(R/S), and shows that antibiotic-resistant bacteria were preferentially stimulated in the rhizospheres of all the plants tested, but most markedly in the rhizospheres of legumes. Also, whereas legumes showed this effect as early as 6 days, plants from other families only showed it when they were quite old (53 days). *Pisum sativum* and *Trifolium repens* were selected for later experiments.

Table 1. *R/S ratios for resistant and non-resistant bacteria for different plant species*

R/S ratio = ratio of number of bacteria in rhizosphere to number of bacteria in corresponding soil. GP = glucose peptone agar. GPStRb = glucose peptone agar + streptomycin + rose bengal.

Age of plant (days)	Plant species	R/S ratio obtained from count on GP agar	R/S ratio obtained from count on GPStRb agar
12	*Phaseolus angularis*	2·8	29·0
12	*P. vulgaris*	19·4	27·0
12	*Vigna unguiculata*	6·4	37·0
6	*Trifolium repens*	6·0	20·0
21	*T. repens*	9·6	57·0
20	*Glycine max*	21·0	866·0
20	*Vicia faba*	16·8	1716·0
12	*Pisum sativum*	4·0	16·0
29	*P. sativum*	61·0	322·0
29	*Triticum vulgare*	32·0	28·0
53	*T. vulgare*	35·0	133·0
29	*Lactuca sativa*	1·8	0·02
53	*L. sativa*	10·0	34·0
29	*Lycopersicum esculentum*	7·7	2·2
53	*L. esculentum*	6·0	21·0
29	*Raphanus sativus*	27·0	23·0
53	*R. sativus*	50·0	270·0

Sources of variation in the examination of rhizosphere and non-rhizosphere samples of soil

Before studying the stimulation of the streptomycin resistant flora in the rhizosphere in detail, variations due to technique and to plant variability were examined.

Replicate variance and validity of the control samples. There were too few replicates in the previous experiments to estimate the sampling variance, and the control samples were from unplanted pots and, therefore, had a different moisture régime from the planted pots. To examine these possible sources of variation, replicate pots of Pastures field soil were each seeded with one pea plant for rhizosphere samples and unseeded pots of soil were used for controls. To obtain control samples from seeded pots, cylinders of fine-mesh heavy-weight Terylene gauze were placed in each pot before sowing. The pea roots did not penetrate the gauze, but the soil in the cylinders was subjected to the same variation in moisture conditions as the soil around the pea roots. The soil samples were taken 30 days after seeding and dilution series prepared and platings made on glucose peptone agar and GPStRb agar. Table 2 shows both the numbers of bacteria and the R/S ratios. The R/S ratio of the resistant bacteria was everywhere higher than that of the total population.

Table 2. *Mean number of resistant bacteria and total bacterial population/g. dry weight soil in rhizosphere and in two sets of control samples*

	Counted on medium					
	GP agar			GPStRb agar		
Plant	Rhizosphere	Pot soil	Cylinder soil	Rhizosphere	Pot soil	Cylinder soil
	Bacteria $\times 10^{-6}$/g. dry soil					
1	194	27·8	17·4	32	0·63	0·17
2	103	15·4	20·0	28	0·50	0·79
3	213	25·0	20·0	29	0·38	0·28
4	261	14·1	17·1	39	0·55	0·26
5	531	15·2	19·1	127	0·35	0·66
6	246	18·5	9·9	41	0·46	0·14
Average	258	19·3	17·2	49·3	0·48	0·38

	R/S ratios from colony counts on			
	GP agar		GPStRb agar	
Plant	Pot soil	Cylinder soil	Pot soil	Cylinder soil
	Bacteria $\times 10^{-6}$/g. dry soil			
1	6·9	11·1	50·8	188·2
2	6·7	5·1	56·0	35·4
3	8·5	10·6	76·3	103·6
4	18·5	15·3	70·9	150·0
5	34·9	27·8	362·8	192·4
6	13·3	24·8	89·1	292·8
Average	14·8	15·7	117·6	160·4

GP = glucose peptone; GPStRb = GP + streptomycin + rose bengal.

For statistical analysis the stimulation of resistant bacteria in the rhizosphere was expressed as the difference between the log values of the rhizosphere and control count on GPStRb agar and the log values of the rhizosphere and control count on glucose peptone agar. These differences were calculated for each of the six replicates and from these the mean difference was obtained. The mean difference with unseeded pots as controls was $+0{\cdot}43 \pm 0{\cdot}031$ and with soil from the Terylene gauze cylinder as control $+0{\cdot}49 \pm 0{\cdot}031$. These differences are individually highly significant, confirming the preferential stimulation of resistant bacteria in the rhizosphere of the pea plants. There was no significant difference in the numbers of either type of bacteria in the two sets of controls, showing that unseeded pots of soil could be used as controls, although the soil was subjected to moisture conditions different from the seeded pots.

The effect of streptomycin and rose bengal separately and together in the medium

In two experiments the effects of streptomycin and rose bengal were examined separately and together. Pea seeds were sown in Pastures field soil and unseeded pots of soil were used as controls. After 30 days, three rhizosphere and three control samples were taken and soil dilutions prepared. Suitable dilutions were then plated with the following agars: glucose peptone; glucose peptone + streptomycin (GPSt); glucose peptone + rose bengal (GPRb); glucose peptone + streptomycin + rose bengal (GPStRb). In another experiment two sets of plants and controls were examined similarly after 22 days. Table 3 shows the results.

Table 3. *The effects of streptomycin and rose bengal, separately and together, on counts of bacteria from rhizosphere and from control soils*

Peas were grown in Pastures field soil; samples taken after 30 days (Expt. 1) and 22 days (Expt. 2). R/S ratio = ratio of number of bacteria in the rhizosphere to number of bacteria in corresponding soil

Sample	GP agar	GPRb agar	GPSt agar	GPStRb agar
	Numbers of bacteria $\times 10^{-6}$/g. dry soil as estimated from colony counts on media			
Expt. 1				
Rhizosphere	478	409	229	55
Control	15·3	14·6	0·8	0·8
	R/S ratio			
	31	28	263	68
	Count $\times 10^{-6}$/g. dry soil			
Expt. 2				
Rhizosphere	284	230	35	70
Control	7·2	5·1	0·5	0·2
	R/S ratio			
	39	45	70	350

GP = glucose peptone; GPRb = GP + rose bengal; GPSt = GP + streptomycin; GPStRb = GP + streptomycin + rose bengal.

The presence of rose bengal alone in the medium had little effect upon the bacterial numbers, either in the soil or rhizosphere, whereas streptomycin alone prevented the development of an appreciable proportion of the population. With streptomycin + rose bengal present the numbers of bacteria/g. dry soil, as estimated from the plates made from soil samples, were affected differently in the two experiments; in Expt. 1 the numbers were the same as with streptomycin alone ($0{\cdot}8 \times 10^6$/g.), but in Expt. 2 they were decreased from $0{\cdot}5 \times 10^6$ to $0{\cdot}2 \times 10^6$/g. Also there was a marked and irregular effect on the rhizosphere count; in Expt. 1 it was decreased from 229×10^6 to 55×10^6, but in Expt. 2 it was increased from 35×10^6 to 70×10^6/g. No explanation can be offered for these divergent effects of supplementary rose bengal.

The fact that the bacteria were unaffected by rose bengal alone indicates that streptomcyin was the operative substance; this was further substantiated by the following tests in which the effects of streptomycin and rose bengal were separately examined on a range of streptomycin-resistant bacteria isolated from GPStRb agar plates. Glucose peptone agar plates were seeded with cultures of the various resistant bacteria and assay cylinders placed on the surface of the plates. Different concentrations of rose bengal (mg./l., 6600, 66, 33, 6·6) were added to one series of cylinders and different concentrations of streptomycin (mg./l., 12,000, 120, 30, 12) added to another series of cylinders. The plates were incubated at 25° for 7 days. All the bacteria were completely inhibited by rose bengal at 6600 mg./l. and some by streptomycin at 12,000 mg./l.; lower concentrations of either substance had no effect. Thus, whatever may be the nature of the action of the streptomycin and rose bengal when together, these bacteria are resistant to both substances and it is reasonable to call the population streptomycin-resistant. The use of rose bengal in the medium may have the disadvantage of increasing the error of estimating the streptomycin-resistant population, but this is offset by its great practical value. Its function in the medium is primarily to restrict fungal growth; without it colonies are exceedingly difficult to count because of fungal overgrowth, particularly at low dilutions. For this reason rose bengal was included in all the media used later.

The selective effect of the basal medium

The glucose peptone agar used in the present work was somewhat selective and, should the streptomycin-resistant population be favoured in preference to others, the preferential stimulation of resistant types would be over-estimated. To examine the effect of the basal medium, rhizosphere and control samples from two sets of pea plants were plated with soil-extract agar, with and without the antibacterial agents; also with glucose peptone agar and GPStRb agar. Soil-extract agar was chosen for this comparison as it was the least selective of the media generally used. Table 4 shows that, as expected, the total count on the soil-extract agar without the antibacterial agents was larger than on glucose peptone agar, but the resistant population counts were similar on both media. Thus the preferential stimulation of resistant bacteria was evident with both media but appeared greater with GPStRb agar because the control resistant-count was lower. Glucose peptone agar was used as the basal medium in all further experiments because the colonies grew better and were easier to count.

Table 4. *Average number of bacteria per g. dry weight of soil as estimated from soil extract and glucose peptone agars with and without antibacterial agents*

	Bacteria × 10^{-6}/g. dry soil					
	Total population			Resistant population		
Medium	Rhizosphere	Control	R/S	Rhizosphere	Control	R/S
Soil extract	400 586	10·0 8·0	40 73	62 61	0·6 0·7	103 87
Mean	493	9·0	55	61·5	0·65	95
Glucose peptone	238 330	8·0 6·3	30 52	57 83	0·2 0·2	285 415
Mean	284	7·1	40	70	0·2	350

The properties of the bacteria

Lochhead & Chase (1943) devised a system of grouping soil bacteria according to certain physiological criteria based on their nutritional requirements, rather than classifying them by biochemical tests, which were inadequate for any 'rational grouping helpful to an understanding of the activity or significance of these bacteria in soil'. They divided the bacteria into seven groups based on their growth in seven media, ranging from those organisms which could grow in a simple basal medium to those requiring unidentified substances present in both yeast and soil extracts. Lochhead & Thexton (1947) showed that those bacteria which grew well in the simple medium were relatively more abundant in the rhizosphere, also that there was an increased incidence of bacteria requiring amino acids for maximum growth or which were stimulated by amino acids. Thus certain nutritional groups of bacteria were preferentially stimulated in the rhizosphere.

It was considered of interest in the current work to know whether the streptomycin-resistant bacteria in the rhizosphere had similar nutritional requirements and could be placed in groups known to be preferentially stimulated, or whether they had widely divergent requirements. They were compared with non-resistant bacteria in the rhizosphere and resistant and non-resistant bacteria in the corresponding soil. Lochhead & Chase's method was used but growth in four media only was examined. These were the basal medium, basal medium + amino acids, basal medium + yeast extract and basal medium + soil extract + yeast extract. Colonies of bacteria were systematically picked, about 60 for each sample, so that all on a plate or sector were taken, and inoculated on to glucose peptone agar and checked for purity. The various nutritional media were then inoculated and after incubation for 5 days at 25° the growth response of each isolate recorded by measuring the turbidity. The relative incidence of the various nutritional groups of bacteria and the estimated number of bacteria of the different groups per g. soil are shown in Table 5.

As expected there was an increased percentage in the rhizosphere of non-resistant bacteria with simple requirements and those needing amino acids. An interesting feature was that the resistant bacteria in the rhizosphere showed a greater percentage which had simple needs and a lower percentage requiring amino acids than did the non-resistant bacteria. This preferential stimulation of bacteria with simple needs was also reflected in the R/S ratio: 125 with resistant bacteria as compared with 30 with non-resistant bacteria. Thus it seems that most of the preferentially stimulated resistant bacteria belonged to one nutritional group.

In addition to this property 85 % of the resistant bacteria were chromogenic, producing a yellow pigment; half of these were identified as a *Flavobacterium* species (*Bergey's Manual*, 1957); the specific identity was not determined. The organism was a Gram-negative non-motile rod which produced a yellow pigment, the hue depending on the medium, being particularly bright on gelatine. Litmus milk was made alkaline; there was no proteolytic activity. Growth occurred in a wide range of carbohydrate media, sometimes with the production of acid, but never of gas; starch was not hydrolysed. The bacterium had simple nutritional requirements. The other chromogenic bacteria were also probably flavobacteria, producing bright yellow-orange pigments, which did not change colour with the medium. The specific identities were not determined. Three strains of these bacteria were found, the first having simple nutritional needs, the second required amino acids and the third required unidentified substances from yeast extract.

Of the non-resistant rhizosphere bacteria, only 33 % were chromogenic and were identified as *Flavobacterium* spp., but with different properties from the resistant types. In the soil 19 % of the resistant organisms and 12 % of the non-resistant types were chromogenic. These were identified as *Flavobacterium* spp.

Table 5. *Nutritional groups of bacteria in rhizosphere and control soil*

	Resistant bacteria				
	Rhizosphere		Control		R/S
	Plate count ($\times 10^{-6}$/g.)				
	3·3		0·36		91
Nutritional groups	% Colonies	Estimated no. ($\times 10^{-6}$/g.)	% Colonies	Estimated no. ($\times 10^{-6}$/g.)	R/S
Grow in basal medium	61	2·0	44	0·016	125
Require amino acids	20	0·7	56	0·02	35
Require yeast extract	18	0·6	—	0	—
Require yeast + soil extracts	—	0	—	0	—

	Non-resistant bacteria				
	Rhizosphere		Control		R/S
	Plate count ($\times 10^{-6}$/g.)				
	184		7·1		26
Nutritional groups	% Colonies	Estimated no. ($\times 10^{-6}$/g.)	% Colonies	Estimated no. ($\times 10^{-6}$/g.)	R/S
Grow in basal medium	50	93	43	3·1	30
Require amino acids	25	46	12	0·9	51
Require yeast extract	20	38	31	2·2	17
Require yeast + soil extracts	4	7	12	0·9	7

DISCUSSION

These experiments showed that bacteria resistant to streptomycin + rose bengal were stimulated to a greater degree than non-resistant bacteria in the rhizosphere of a variety of plant species. The stimulation of resistant bacteria by legumes was greater than that produced by non-legumes, a result which was consistent with present knowledge concerning the legume rhizosphere. Also, this stimulation occurred with younger plants than with non-legumes, which might be related to the faster release of stimulatory substances; for example, Rovira (1956) showed that pea roots excreted greater amounts of amino acids during the first 21 days of growth than did oat roots. However, most of the streptomycin-resistant organisms had simple nutritional requirements, which suggested that, although amino acids might stimulate, they were not obligatory for these bacteria and the preferential stimulation of this group was not entirely dependent upon the presence of amino acids in the rhizosphere. Lochhead (1948) noted that there was a preferential stimulation of chromogenic bacteria in the rhizospheres of a variety of crop plants; in the current work, also, the streptomycin-resistant bacteria were mostly chromogenic, the predominant form being a *Flavobacterium* species. A possible reason for the stimulation of streptomycin-resistant bacteria in the rhizosphere might lie in their property of antibiotic resistance. Bacteria, fungi and actinomycetes are all stimulated in the rhizosphere and species of all these can produce antibiotics; it has been suggested (Brian, 1957) that the rhizosphere is a region where such production could take place. Thus bacteria resistant to antibiotics would have a competitive advantage over those which were sensitive, and such a group could be preferentially stimulated. Further examination of such a relationship between the various groups of soil micro-organisms in the rhizosphere is needed.

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Chemical and Metabolic Properties of Various Elements Found in Cultures of a Stable Proteus L Form

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SUMMARY

The microscopic elements which constituted cultures of a stable L form (Proteus L9) were found to be highly heterogeneous with respect to morphological, chemical and metabolic properties. The smallest elements found in the cultures studied (diameter $< 0{\cdot}3\mu$) contained more lipid-phosphorus (lipid-P), but less ribonucleic acid-phosphorus, deoxyribonucleic acid-phosphorus and protein-nitrogen, than whole cultures consisting predominantly of bodies of diameter $> 1\mu$. The small elements respired at about the same rate as whole cultures, but they showed low, if any, biosynthetic activity. The small elements probably possess a structural organization similar to that of L bodies of larger sizes, but most of them contained little, if any DNA.

INTRODUCTION

The bacterial L forms (reviewed by Dienes & Weinberger, 1951; Tulasne, 1955; Kandler & Kandler, 1960; Kleineberger-Nobel, 1960) are highly pleomorphic. Vesicular and granular elements are most frequent in cultures of L forms, but various irregular structures may also be found. Mandel and his associates have published a series of papers describing the fractionation of L cultures into fractions characterized among other things by the size of the elements present in them (Mandel, Terranova & Sensenbrenner, 1957; Mandel, Feo, Sensenbrenner & Terranova, 1959; Mandel, Terranova, Sensenbrenner & Feo, 1959; Mandel *et al.* 1959). The fractionation was performed by means of differential centrifugation. This technique has been used in most of the experiments described in the present paper. A stable L form derived from *Proteus mirabilis* was investigated.

METHODS

Organism. The Proteus L form used was obtained from Dr E. Klieneberger-Nobel (Lister Institute of Preventive Medicine, London). It has not shown any signs of reversion during its history and has been designated as strain L9 (Klieneberger-Nobel, 1956). Tests performed in our laboratory have shown that the normal Proteus strain from which Proteus L9 was derived does not produce acid and gas from maltose, and does not produce indole; this strain thus exhibits the properties of *Proteus mirabilis* (see Taubeneck, 1956).

Growth conditions and harvesting of the cultures. Samples of the L form were obtained for experimental purposes from stock cultures grown in 250 ml. Erlenmeyer flasks containing 50 ml. of the liquid serum-free medium described by

Abrams (1955). No penicillin, however, was included in the medium used in the present work. The culture were incubated for 24 hr. at 30° on a rotary shaker (100 rev./min.) and stored at room temperature. Subcultures were made every week.

To obtain large amounts of L form grown in a liquid medium, 3 l. Erlenmeyer flasks containing 500 ml. medium were used. The flasks were inoculated with 50 ml. of an overnight culture of Proteus L9, obtained as described above.

For the growth of Proteus L9 on solid medium, Petri dishes (diam. 20 cm.) were used containing Abram's liquid medium supplemented with 0·8% (w/v) Difco agar. The plates were heavily inoculated with a liquid culture of Proteus L9 and incubated for 48 hr. at 30°. For harvesting the L bodies, a liquid culture of Proteus L9 was centrifuged for 20 min. at 78,000 *g*. The supernatant fluid was used for suspending the growth present on the Petri plates.

Differential centrifugation. The centrifugations were made with a Spinco model L preparative ultracentrifuge equipped with a no. 30 rotor (tube diameter 2·54 cm., tubes inclined 26° from axis of rotation). In each fractionation experiment the centrifugations were conducted in two centrifugal fields, as outlined in Fig. 1. All centrifugations lasted for 20 min., plus the time required for stopping the rotor with the magnetic brake.

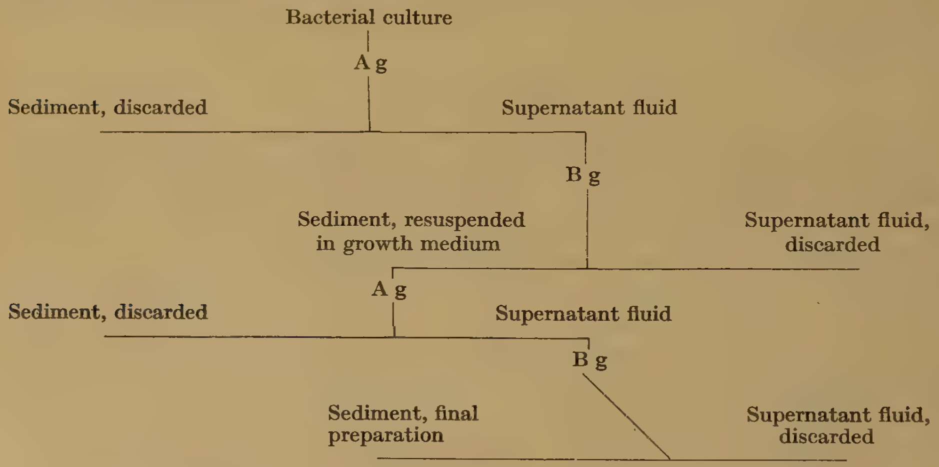
Bacterial culture
A g
Sediment, discarded
Supernatant fluid
B g
Sediment, resuspended in growth medium
Supernatant fluid, discarded
A g
Sediment, discarded
Supernatant fluid
B g
Sediment, final preparation
Supernatant fluid, discarded

Fig. 1. Fractionation of L elements by means of differential centrifugation. The symbols 'A g' and B g' stand for the centrifugal fields used.

Chemical analyses. For the determination of lipid-phosphorus, nucleic acids and protein, samples were subjected to the Schneider fractionation procedure (Schneider, 1945; Weibull & Beckman, 1960). Total phosphorus in the lipid fraction (lipid-P) was determined according to Allen (1940). Ribonucleic acid-phosphorus (RNA-P) was determined as described by Schneider (1945) and deoxyribonucleic acid-phosphorus (DNA-P) according to Burton (1956). The Kjeldahl method was used to estimate protein nitrogen (protein-N). The dry weight of bacterial material was determined as described by Weibull & Beckman (1960).

Enzyme assays and measurements of respiration. Succinic dehydrogenase was determined according to Storck & Wachsman (1957). Catalase was estimated by the method described by Bonnichsen, Chance & Theorell (1947). These measurements were made at room temperature. The conventional Warburg technique was used for respiration measurements. The experiments were conducted at 30°.

Isotope experiments. ^{32}P was obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England. Activities were measured with a Tracerlab TGC-2 Geiger tube, connected to a Tracerlab SC33A '1000' Scaler. Samples were measured either wet (volume of sample 1 ml.) or after drying at 100°.

RESULTS

Fractionation by means of filtration

The experiments described in this section were performed with Proteus L9 grown in Abram's liquid medium. Microscopical inspection revealed spherical bodies of various sizes as predominant constituents of these cultures (see Weibull & Beckman, 1960); minor amounts of morphologically ill-defined material were also seen. The spherical bodies were single, or formed small aggregates, each consisting usually of less than 10 individual spheres. When the L cultures were centrifuged at high speed in the Spinco centrifuge, a sediment consisting of two rather well-defined layers was usually formed. The upper one was transparent, the lower one opaque. However, when the two layers were resuspended separately in the growth medium, the suspensions obtained could not be clearly distinguished from each other when viewed by phase-contrast microscope.

Up to about half of the bacterial material of a Proteus L9 culture did not pass through a Munktell 1F filter paper (manufactured by Grycksbo Pappersbruk, Ltd, Grycksbo, Sweden). When the filtrate was centrifuged, the opaque material mentioned above was almost completely absent from the centrifugal pellet. The filtered bacterial material always contained more lipid-P and nucleic acid phosphorus per mg. dry weight than the corresponding unfiltered material. On the other hand, the content of protein-N was about the same in the two kinds of material. Thus the ratio content of lipid-P, RNA-P, DNA-P, protein-N, in 1 g. dry wt. filtered material to content of same substance in 1 g. unfiltered material was found to be: lipid-P, 1·36; RNA-P, 1·36; DNA-P, 1·41; protein-N, 0·94. These figures represent average values obtained from four separate experiments.

The respiration of filtered L cultures was found to be about 30 % more vigorous than that of unfiltered material, calculated per mg. bacterial dry weight.

Fractionation by means of differential centrifugation

The main purpose of these experiments was to isolate from L cultures small elements similar to those described by Tulasne (1955) and to study the chemical and metabolic properties of these elements as compared with those of the unfractionated cultures. Cultures grown in a liquid medium and on a solid medium (see Methods) were investigated. The type of medium did not influence the results markedly. However, larger yields of bacterial material were obtained when Proteus L9 was grown on a solid medium. When not otherwise mentioned, the data reported below refer to experiments carried out with solid medium.

Morphological studies. As with L cultures grown in liquid medium, unfractionated cultures grown on solid medium consisted essentially of spherical elements of various sizes. Electron microscopical observations showed that the fractions obtained by differential centrifugation contained essentially elements having a diameter $< 0{\cdot}3\,\mu$.

Chemical studies. Unfractionated and fractionated L cultures were analysed for lipid-P, protein-N, RNA-P and DNA-P. Table 1 gives data from a number of independent experiments. The gravitational fields applied for fractionation of the L cultures differed from experiment to experiment, ranging from 5500 to 78,000 *g*. The fractions were examined by oil-immersion phase-contrast microscopy; this revealed granular elements of a size near the resolving limit of the microscope as predominant elements in all the fractions obtained.

The figures collected in Table 1 show that, on a dry-weight basis, the unfractionated L bodies contained on an average more RNA-P, DNA-P and protein-N than the small elements isolated from them, but less lipid-P. The wide variations in the DNA-P content of the small bodies should be emphasized; the maximal and minimal values differed by a factor of 64. The corresponding factor for the other compounds assayed varied between 1·2 and 4·8.

Table 1. *Chemical composition of unfractionated L cultures and of fractions of such cultures obtained by differential centrifugation at* 5500–78,000 *g*

All data are based on results obtained from 5 to 10 independent experiments. The ± sign indicates standard error.

Substance assayed		Unfractionated cultures	Small elements (diameter $< 0{\cdot}3\,\mu$) obtained by differential centrifugation
		(% dry wt. of material analysed)	
Lipid-P	Max. and min. value	0·51, 0·71	0·72, 1·24
	Average	0·57 ± 0·04	1·01 ± 0·11
RNA-P	Max. and min. value	0·55, 0·83	0·21, 1·00
	Average	0·71 + 0·06	0·45 ± 0·07
DNA-P	Max and min. value	0·35, 0·43	0·01, 0·64
	Average	0·39 ± 0·02	0·18 ± 0·06
Protein-N	Max. and min. value	8·18, 9·79	4·90, 6·91
	Average	8·61 ± 0·31	6·32 ± 0·41

The pronounced fluctuations in the DNA content of the fractionated L cultures suggest that at least part of the DNA did not form an integral part of the spherical bodies in these preparations. To test this hypothesis crystalline DNAse (Mann Research Laboratories Inc., New York 6, N.Y., U.S.A.) was added to a suspension of small L elements in the growth medium. The mixture was incubated for 2 hr. at 30° and then analysed for lipid-P, RNA-P and DNA-P; an unfractionated culture containing mainly elements having a diameter $> 1\,\mu$ was similarly treated. This culture had been grown in liquid medium, since L bodies grown on solid medium have a tendency to lyse when suspended in a fluid and then probably release DNA.

Table 2 shows the results of the digestion experiment. It can be seen that the chemical composition of the unfractionated culture was not influenced by the enzymic treatment. The content of lipid-P and RNA-P in the small elements remained practically constant during the treatment with DNAse. The DNA-P content, on the other hand, diminished by about 70 %.

Table 2. *Digestion with DNAse of an unfractionated L culture and of a fraction of such a culture obtained by means of differential centrifugation*

The fractionated preparation contained only small elements (diameter < 0·3 μ); the unfractionated culture mainly elements of diameter > 1 μ. The digestion took place in the growth medium at 30° for 2 hr. The concentration of DNAse was 0·5 mg./ml., that of the L elements about 1 mg. dry wt./ml.

Substance assayed	Unfractionated L culture: Before digestion	Unfractionated L culture: After digestion	Fraction containing small L elements: Before digestion	Fraction containing small L elements: After digestion
	% dry wt. material analysed			
Lipid-P	0·60	0·60	1·15	1·14
RNA-P	0·68	0·69	0·41	0·42
DNA-P	0·36	0·36	0·28	0·09

As mentioned in Table 1 the data about small L elements refer to batches obtained by centrifugation at different centrifugal fields. To test the relationship between the centrifugal fields applied and the chemical composition of the fractions obtained, some L cultures were fractionated into two or three fractions. Each fraction was isolated as outlined in Fig. 1 and was analysed chemically. Table 3 shows the results of three experiments of this kind. It can be seen that the RNA-P content of elements collected by using high centrifugal fields tended to be lower than that of elements obtained by centrifugation at lower speeds. The content of DNA-P, lipid-P and protein-N in the fractions analysed varied less, or in an irregular manner.

Table 3. *Chemical composition of L elements isolated by means of differential centrifugation at various centrifugal fields*

The centrifugations were carried out as outlined in Fig. 1.

Expt.	Applied centrifugal fields (*g*)	Lipid-P	RNA-P	DNA-P	Protein N
		(% dry weight of the bacterial material analysed)			
I	2,100, 5,500	—	0·54	0·016	—
	5,500, 19,600	—	0·43	0·020	—
	19,600, 78,000	—	0·21	0·008	—
II	2,100, 5,500	—	0·39	0·068	—
	5,500, 19,600	—	0·28	0·038	—
III	2,100, 5,500	0·65	0·50	0·17	6·91
	5,500, 19,600	0·80	0·51	0·20	6·68

To compare the structural and macromolecular organization of L bodies of various sizes, unfractionated and fractionated L cultures were shaken in a Mickle disintegrator (Mickle, 1948) for 2 min. After this treatment only bodies or fragments of a size near the resolving power of the light microscope were seen in the cultures. The broken material was centrifuged at 103,000 *g* for 20 min. The supernatant liquid obtained was centrifuged for a further 120 min. at 103,000*g*, transferred to fresh tubes and again centrifuged at the same speed for a further 120 min. Table 4 shows the distribution of lipid-P, RNA-P and DNA-P in the fractions obtained by this centrifugation procedure. It can be seen that the greater part of the lipid-P of both whole L cultures (mainly containing bodies with a diameter > 1 μ) and small L bodies (diameter < 0·3 μ) was sedimented when the disintegrated L bodies were centrifuged at 103,000*g* for 20 min. Most of the DNA-P was sedimented after prolonged centrifugation at the same speed. The RNA-P of the whole L cultures and the small L bodies behaved differently when the disintegrated cultures were centrifuged. Thus about 80 % of the RNA-P of the small L bodies was sedimented after centrifugation at 103,000 *g* for 20 min., whereas about half of the RNA-P of unfractionated cultures was not sedimented even after centrifugation at the same speed for 260 min.

Table 4. *Distribution of lipid-P, RNA-P and DNA-P in fractions of L bodies obtained by disruption of these bodies in a Mickle disintegrator and differential centrifugation of the disintegrated material*

Each figure represents an average value from two independent experiments.

		Cell material sedimented by centrifugation at		Cell material, not sedimented by centrifugation at 103,000 g for 260 min.
		103,000 *g* for 20 min.	103,000 *g* for 260 min. (20 + 120 + 120)	
		% of that found before centrifugation		
Lipid-P	Whole L cultures	75·3	15·6	10·1
	Small L bodies (diameter < 0·3 μ)	82·2	16·0	5·2
RNA-P	Whole L cultures	21·4	38·1	41·9
	Small L bodies (diameter < 0·3 μ)	81·4	15·0	6·5
DNA-P	Whole L cultures	16·6	72·1	16·4
	Small L bodies (diameter < 0·3 μ)	11·4	64·0	28·4

Enzyme studies. Many enzymes of the bacterial cell are undoubtedly located in definite subcellular structures (Marr, 1960). To elucidate the nature of the small bodies (diameter < 0·3 μ) isolated from whole L cultures by means of differential centrifugation, some enzymic activities of these bodies were studied and compared with those of unfractionated cultures. Succinic dehydrogenase and catalase were the two enzymes chosen for this investigation since they are located differently, at least in some bacteria (Alexander & Wilson, 1955; Weibull, Beckman & Bergström, 1959). The amounts of succinic dehydrogenase and catalase found in unfractionated cultures of Proteus L9 grown on a solid medium, and of fractions of such cultures obtained by means of differential centrifugation were: succinic dehydrogenase, 20·8 ± 1·6 and 35·0 ± 2·1 units; catalase 70,000 ± 3200 and 20,100 ± 2600 units (the enzymic activities are expressed as μmole of substrate oxidized or split per min. and per l. of an incubation mixture containing 1 g. dry weight bacterial material).

Thus the unfractionated L bodies contained more catalase but less succinic dehydrogenase than the fractionated material.

The localization of succinic dehydrogenase and catalase in L bodies of various sizes was investigated by treating unfractionated and fractionated L cultures in a Mickle disintegrator and centrifuging the broken material in the manner used for characterization of the macromolecular organization of the L bodies by means of chemical analyses (see the preceding section of this paper); Table 5 shows the results of these experiments. It can be seen that both the catalase and the succinic dehydrogenase of the L bodies were distributed in much the same manner in unfractionated L cultures (containing mainly elements having a diameter $> 1\mu$) and in small L bodies (diameter $< 0{\cdot}3\mu$). The succinic dehydrogenase was associated with sedimentable matter, whereas the major part of the catalase was not sedimented even after prolonged centrifugation at high gravitational fields.

Table 5. *Distribution of succinic dehydrogenase and catalase in fractions of L bodies obtained by disruption of these bodies in a Mickle disintegrator and differential centrifugation of the disintegrated material*

The results are expressed as per cent of total enzymic activity of the material before the centrifugation procedure

		Cell material sedimented by centrifugation at		Cell material not sedimented by centrifugation at 103,000 *g* for 260 min.
		103,000 *g* for 20 min.	103,000 *g* for 260 min. (20 + 120 + 120)	
Succinic dehydrogenase	Whole cultures	72·1	< 1	< 1
	Small L bodies (diameter $< 0{\cdot}3\mu$)	69·0	< 1	< 1
Catalase	Whole L cultures	1·0	30·4	52·0
	Small L bodies (diameter $< 0{\cdot}3\mu$)	5·0	29·6	48·0

Respiration experiments. Measurements were carried out on unfractionated L cultures grown in liquid and on solid media. Fractions of L cultures, obtained by differential centrifugation and containing as predominant elements bodies of a diameter $< 0{\cdot}3\mu$, were also studied. The growth medium served as substrate. The Q_{O_2} values obtained were: unfractionated L culture, grown in liquid medium, 410 ± 52; similar culture, treated for 2 min. in a Mickle apparatus, < 5; unfractionated L culture, grown on solid medium, 233 ± 31; fractionated L elements, 209 ± 26. Thus unfractionated L cultures grown in liquid medium respired about twice as vigorously as L bodies grown on solid medium. On the other hand, fractions obtained by differential centrifugation respired at about the same speed as did the whole cultures from which they were prepared (the fractionated material was obtained from cultures grown on a solid medium). Mechanical disintegration abolished the respiration of the L bodies almost completely. The same result was obtained when L bodies were ruptured by suspending them in distilled water. No oxygen consumption was observed when L bodies were suspended in Krebs–Ringer solution containing glucose (see Mandel, Terranova, Sensenbrenner & Feo, 1959). No certain relationship was detected between the chemical constitution of fractionated L cultures and their respiratory activity. In Fig. 2 the circles in the diagram indicate the DNA-content of several batches of fractionated material and the corresponding Q_{O_2} values.

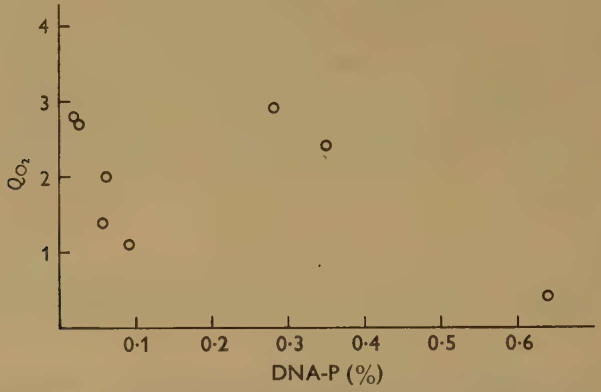

Fig. 2. Q_{O_2} values and DNA-P content of fractionated L cultures, predominantly consisting of spherical bodies of a diameter smaller than 0·3 μ. The fractionation was carried out by means of differential centrifugation.

Estimations of the number of viable elements in unfractionated and fractionated L cultures. These estimations to be described in detail elsewhere were made by the pour-plate method. The nature of the growth medium used was of decisive importance for the outcome of the experiments. Thus in the case of unfractionated L cultures, no growth was obtained in pour plates when fresh Abram's medium was used for diluting the cultures and for pouring the final dilutions. However, a growth medium which gave viable counts not very much lower than the corresponding total counts as determined according to Weibull (1960), was obtained by centrifuging an overnight culture of Proteus L9 and sterilizing the supernatant fluid by heating at 56° for 1 hr. The inclusion of 10 % (v/v) horse serum in this heated supernatant fluid further slightly increased the viable counts.

When fractionated L cultures, containing as predominant constituents bodies of diameter $< 0{\cdot}3\,\mu$, were tested for viable counts on the serum-containing medium described above, at the most 5×10^5 colonies were obtained per mg. dry wt. fractionated material. It can be calculated that this material must have contained more than 10^{11} individual L bodies (diameter $< 0{\cdot}3\,\mu$). This implies that the number of non-viable bodies would exceed that of the viable ones by a factor greater than 10^5. When fractionated L cultures were investigated the viable counts obtained could thus be attributed to large L elements, present as contaminants. Because of their scarcity such elements might easily be overlooked in microscopic preparations.

The biosynthetic activity of small L bodies

The experiments described in the preceding section showed that of the small L bodies (diameter $< 0{\cdot}3\,\mu$) isolated by differential centrifugation, at the most very few ($< 0{\cdot}001$ %) were able to grow, divide and finally to form visible colonies under the experimental conditions used. It might be argued, however, that these bodies might exhibit less extensive biosynthetic activity. To test for this, small L elements were incubated in a growth medium for 2–4 hr. at 30°. At the beginning of the experiment, ^{32}P was added to the incubation mixture at a final concentration of about 15 μC/ml. Samples were taken during the incubation and fractionated according to the Schneider procedure. The incorporation of ^{32}P in the nucleic acid fraction of the L elements during the incubation was followed by radiochemical analyses. The net changes in the content of lipid-P, RNA-P, DNA-P and protein-N of the L elements were followed by conventional chemical analyses (Schneider, 1945; Burton, 1956).

Three experiments of this kind were performed with different growth media. In the first experiment (indicated by the Roman numeral I in Fig. 4) fresh Abrams's medium (modified as described in the next paragraph) was used, supplemented by about half its volume of a batch of Abrams's medium which had been previously used for growing L bodies and then centrifuged and sterilized (this medium will subsequently be called 'old' Abrams's medium). In the second experiment (II)

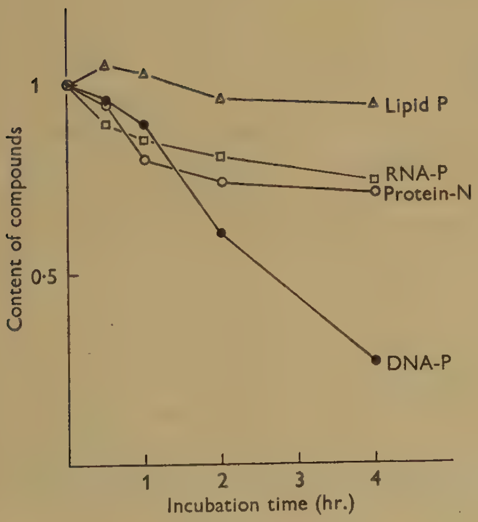

Fig. 3

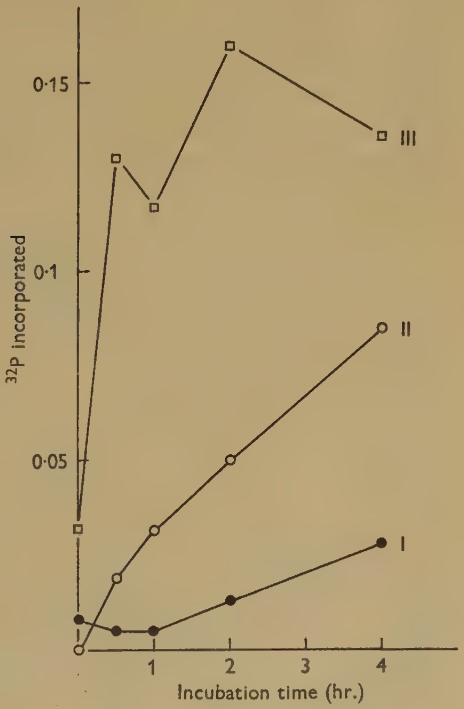

Fig. 4

Fig. 3. Content of lipid-P, protein-N, RNA-P and DNA-P in a fractionated L culture incubated in Abrams's medium supplemented with serum. The content of each compound in the L culture at the beginning of the experiment is given as being equal to unity.

Fig. 4. Incorporation of ^{32}P in fractionated L cultures, containing as predominant elements spherical bodies of diameter $< 0{\cdot}3\mu$. The amount of incorporated ^{32}P is expressed as % of nucleic acid-P of the whole culture. The results of three independent experiments (I–III) are given. For experimental details see text.

only 'old' Abrams's medium was used; in the third experiment (III) 'old' Abrams's medium, +10% (v/v) horse serum inactivated by heating for 30 min. at 56°. Thus media were used which gave high viable counts for unfractionated L cultures (see the preceding section).

In all three experiments the phosphate content of the medium was reduced to 2% of the figure given by Abrams (1955) to minimize the amount of added ^{32}P. The tonicity of the medium was restored by the addition of 11·4 g. KCl/l. medium.

In none of the experiments did chemical analysis show any steady net increase in the content of lipid-P, RNA-P, DNA-P or protein-N in the small L bodies during the incubation (4 hr.). Instead, a decrease in the content of high molecular weight compounds in these bodies was generally noticed. Fig. 3 shows the results of an experiment carried out with serum added to the medium. When a similar experiment was made with an unfractionated L culture the content of nucleic acid-P and lipid-P in the culture increased 2–3 times.

Before measuring the radioactivity of the nucleic acid-containing extracts of the fractionated L elements, 0·5 ml. 10N-sulphuric acid, 0·5 ml. 10% (w/v) ammonium molybdate and 3 ml. of a mixture of equal volumes of isobutanol and benzene was added to 2 ml. samples of these extracts. The resulting two-phase systems were shaken for 15 sec. In this way, any inorganic phosphate originally adsorbed to the L bodies would be removed from the aqueous extracts (see Lindberg & Ernster, 1956). At the same time any inorganic phosphate formed by acid hydrolysis of RNA would also be removed from these extracts. The remaining organic phosphate would, however, correspond to all of the DNA-P and about 50% of the RNA-P of the bacterial material analysed (Loring, 1955).

The results of the radiochemical measurements performed are given in Fig. 4. It can be seen that the ^{32}P incorporated in the L elements during the incubation corresponded at the most to between 0·1 and 0·2% of the nucleic acid-P in the L cultures investigated. The possibility cannot be excluded that this small amount of incorporated phosphorus could be attributed to large L bodies present as contaminants in the fractionated L cultures (compare the determinations of viable counts for fractionated L cultures, described in the preceding section).

DISCUSSION

The present work shows the marked heterogeneity of bacterial L cultures. Our fractionation experiments, carried out by means of filtration or differential centrifugation, showed that various fractions characterized by different morphological, chemical and metabolic properties, could be prepared from the cultures of Proteus L9 studied. Most of the fractions prepared contained spherical bodies of different sizes as predominant elements. In the present work most attention was paid to L bodies which had a diameter $< 0{\cdot}3\mu$, which we separated from other elements in cultures of Proteus L9 by differential centrifugation. Light microscope and electron microscope observations showed that larger elements were only occasionally found in the fractionated material thus obtained.

In some bacteria at least the main part of the phospholipid is bound to particulate matter, located in the cytoplasmic membrane (cell envelope) of the intact organism (Mitchell & Moyle, 1951; Marr & Cota-Robles, 1957; Weibull, 1957). A similar distribution seems to characterize the bacterial succinic dehydrogenase (Mitchell & Moyle, 1956; Storck & Wachsman, 1957; Weibull, Beckman & Bergström, 1959). Catalase, on the other hand, generally seems to be a soluble enzyme, probably located within the bacterial cell (Alexander & Wilson, 1955; Few, Frazer & Gilby, 1957; Weibull, Beckman & Bergström, 1959). Most of the RNA of the bacterial cell is also found in the non-peripheral part of the cytoplasm, the cytoplasmic membrane being almost free from this substance (Marr & Cota-Robles, 1957; Gilby, Few & McQuillen, 1958; Weibull, Beckman & Bergström, 1959).

Those of our chemical and enzymic studies which were carried out with mechanically disintegrated L bodies (see Tables 4 and 5) showed that also in Proteus L9 the phospholipid and the succinic dehydrogenase were largely bound to particulate matter and that the major part of the catalase behaved as a soluble enzyme. This was true of unfractionated and fractionated L cultures. Thus at least in some respects the L form investigated seems to possess a structural organization similar to that found in several normal bacteria.

The RNA of small L bodies (diameter $< 0{\cdot}3\mu$) was much more readily sedimented than that of unfractionated cultures consisting mainly of bodies of diameter $> 1\mu$ (see Table 4). Judging from the digestion experiment with DNAse (Table 2), the small L bodies seemed to contain little if any DNA. In other respects, results of the fractionation and disintegration experiments suggest that the structure of the L bodies was about the same, irrespective of size. Thus the small L bodies contained about double the amount of lipid-P than did the unfractionated cultures, and about 40 % less RNA-P (Table 1). Furthermore, our enzyme studies showed that the small L bodies contained more succinic dehydrogenase and less catalase than unfractionated cultures of Proteus L9. Taking into account the greater surface/volume ratio of the small elements as compared with the larger ones, these facts could be explained at least qualitatively if it were assumed that the L bodies consisted of a peripheral layer (the cytoplasmic membrane) containing the major part of the phospholipid and succinic dehydrogenase, and a central part containing the RNA and catalase.

The respiratory experiments showed that the small L bodies respired as vigorously as the unfractionated ones from which they were derived, and that the respiration rate of L bodies grown on solid medium was about 50 % lower than that of L bodies grown in liquid medium.

The work thus demonstrates that L bodies of different sizes differed in their chemical and metabolic properties. This is in agreement with the results of Mandel and co-workers (Mandel, Feo, Sensenbrenner & Terranova, 1959; Mandel *et al.* 1959). Our work has shown, however, that even small L bodies respire under appropriate conditions (cf. Mandel, Terranova, Sensenbrenner & Feo, 1959).

More fundamental differences between L bodies of different sizes than those discussed above appear when the biosynthetic activities are compared. The viable counts were very low for fractionated L cultures which contained as predominant elements small bodies of diameter $< 0{\cdot}3\mu$, the counts amounting to about 0·001 % of the total number of individual elements in the samples studied. Probably the viable elements in the fractionated material consisted of large L bodies, undetected in microscopic preparations of this material because of their scarcity.

Judging from the incorporation experiments performed, the synthesis of nucleic acids in small L elements suspended in a growth medium and incubated for 4 hr. amounted at most to about 0·1–0·2 % of the total nucleic acid content of these elements. Under similar conditions the nucleic acid content of unfractionated L cultures increased 2–3 times. Thus the nucleic acid synthesis in the small L bodies was more than 1000 times less than in whole L cultures; however, the role of contaminating L bodies of larger sizes has to be taken into account. It then seems possible that all the nucleic acid synthesis which occurred in the fractionated material might be attributed to such contaminants. The possible occurrence of exchange reactions should also be borne in mind. The biosynthetic activity of the small L elements seems therefore questionable, as judged from the data presented here. Consequently their role in the life cycle of the L cultures, as described by Tulasne (1955), seems doubtful. On the other hand, these small bodies seems to possess a certain degree of structural organization, and they can hardly be regarded merely as protoplasmic debris. This is especially evident from the fact that mechanically prepared debris of Proteus L9 showed at most a very low degree of respiratory activity, whereas small L bodies isolated by differential centrifugation respired about as intensely as unfractionated L cultures. The chemical and enzymic composition of the small L bodies also suggests a certain degree of structural organization. Earlier work about the structure of small L elements as seen by the electron microscope should also be mentioned in this connexion (Thorsson & Weibull, 1958).

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The Effect of Temperature on the Production by Coagulase-Positive Staphylococci of Staphylokinase and other Extracellular Substances

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SUMMARY

Three strains of coagulase-positive staphylococci, grown at temperatures between 35° and 43°, showed no significant change in mass of growth or in yield of α-haemolysin and the Panton-Valentine (PV) leucocidin. Staphylokinase production, however, decreased by 18 % (per mg. dry weight) for each degree rise in temperature. The amount of hyaluronidase produced at 37° was five to eight times greater than that produced at 41°.

INTRODUCTION

The optimal temperature for growth of a pathogenic organism is usually that of its host. The effects of different temperatures during the growth of numerous micro-organisms has been studied. For example, toxin production by *Corynebacterium diphtheriae* is best at 34°. *Escherichia coli* grown at progressively higher temperatures has its generation time progressively increased (Barber, 1908). *Mycobacterium ulcerans* shows little or no growth above 33° (MacCallum, Tolhurst, Buckle & Sissons, 1948). *Pasteurella pestis* at temperatures above 32° has its synthesis of metabolites markedly depressed (Hills & Spurr, 1952). *P. pseudotuberculosis*, which is strongly motile and possesses good flagellar antigens at 22° loses these at 37° (Preston & Maitland, 1952). *Listerella monocytogenes* produces better flagellar antigens at 25° than at 37° (Paterson, 1939, quoting McGaughey). Type III strains of Pneumococcus which produce only local lesions in rabbits do not survive when grown at 41°, whereas those strains which withstand this growth temperature cause fatal infections (Enders & Shaffer, 1936; Rich & McKee, 1936). It was of interest, therefore, to see what effects, if any, the incubation temperature had with three strains of Staphylococcus on the crop of organisms and yield of staphylokinase, α-haemolysin, Panton-Valentine (PV) leucocidin (Panton & Valentine, 1932) and hyaluronidase.

METHODS

Organisms. Three coagulase-positive strains of Staphylococcus were used in these investigations: P80, a phage-type 80 strain (from the late Dr E. S. Duthie); V8, isolated from a case of chronic furunculosis (Gladstone & Van Heyningen, 1957); the Oxford strain used in the assay of penicillin. The organisms for inoculation were grown on Difco heart infusion agar slopes overnight at 37°. These slopes were kept at room temperature and used during about 4 weeks, after which new slopes were used.

Inoculum. In all cases a 10 % (v/v) inoculum was used, grown in the same medium and at the same temperature as in the test. An overnight culture on a Difco agar slope was inoculated into 10 ml. broth, grown overnight and diluted 1/10 in the test medium.

Production of staphylokinase, PV leucocidin and hyaluronidase. Preliminary tests showed that the same media and cultural conditions were suitable for the production of these substances, but different times of incubation were required. The medium (CCY) was Woodin's modification (Woodin, 1959) of the yeast casein medium of Gladstone & Fildes (1940), namely: Difco Casamino acids, 20 g., Na lactate 70 % syrup, 14·4 ml.; Na glycerophosphate, 20 g.; $MgSO_4.7H_2O$ solution (16 %, w/v), 0·25 ml.; $MnSO_4.4H_2O$ solution (6·4 %, w/v), 0·125 ml.; $FeSO_4.7H_2O$ solution (0·32 %, w/v), citric acid solution (0·32 %, w/v), 2 ml.; KH_2PO_4, 0·41 g.; $Na_2HPO_4.2H_2O$, 3·1 g.; Oxoid yeast diffusate from 20 g. (this yeast diffusate was prepared by dialysing 100 g. yeast extract (Oxoid) dissolved in 100 ml. distilled water against 900 ml. distilled water for 24 hr. in the cold). Distilled water was added to make the final volume of the medium to 1 l. after adjustment to pH 7·6. The medium was distributed in 10 ml. volumes in ⊥-tubes (Van Heyningen & Gladstone, 1953) and autoclaved for 15 min. at 120°. Cultures were shaken exposed to air in a water-bath at a known constant temperature (±0·1°) and were harvested, unless otherwise stated, at 6½ hr. for staphylokinase (time for optimal production; see Results), 14 hr. for hyaluronidase and 18 hr. for PV leucocidin.

Production of α-haemolysin. Preliminary tests showed that 2 % (w/v) Difco heart infusion broth, without the addition of 0·2 % (w/v) agar, produced good yields of α-haemolysin. This medium was distributed in 15 ml. volumes in 4 oz. medical flats and autoclaved at 120° for 15 min. After inoculation, the cultures were gassed with a mixture of 20 % (v/v) CO_2 + 80 % (v/v) air, closed with screw caps and incubated in a horizontal position in a water-bath at known temperature for 48 hr.

Estimation of growth. Growth was estimated in a Hilger Spekker spectrophotometer calibrated in terms of bacterial dry weight per ml. culture.

Harvesting of cultures. After incubation for the appropriate time, the cultures were centrifuged at 2500 rev./min. for 10 min.; the supernatant fluids were retained and used without filtration, and the deposits examined by Gram staining to test for purity. For the estimations of staphylokinase, 1/100,000 thiomersalate was added to prevent further growth.

Assay of staphylokinase

Staphylokinase was assayed on a semi-microscale, by a combining test with antibody, as follows. Glass coverslips were thoroughly cleaned in chromic acid (40 g. potassium dichromate in 1 l. concentrated sulphuric acid) rinsed in tap water and distilled water and stored in ethanol until required. All tubes used for dilutions were cleaned in a similar manner. Four small wax circles were made on each coverslip and into each circle was pipetted a solution of 0·01 % (w/v) fibrinogen (Blood Products Laboratory, Lister Institute) in 0·5 % (w/v) gelatin saline (pH 7·6) using a microsyringe. To each circle was added about 0·002 ml. of a 0·2 % (w/v) solution of thrombin (Blood Products Laboratory, Lister Institute) in gelatin saline containing 0·01 ml. Hucker–Conn fibrin stain/ml. As each drop was added, the two solutions within a circle were mixed with a small glass rod. These quantities were found to provide a firm clot after incubation at 37° for 10 min. in moist chambers.

The minimum lytic dose (MLyD) of the staphylokinase preparation was first determined as follows. The culture supernatant fluid was serially diluted at 100 % differences in gelatin saline. A volume of 0·01 ml. of each dilution, ranging from undiluted supernatant fluid to 1/1024 dilution, was pipetted on to a preformed fibrin clot, prepared as above, by using a microsyringe. As a control, 0·01 ml. of 1/10 dilution of a preparation of soya bean trypsin inhibitor (from Worthington Biochemical Sales Co., New Jersey) was mixed with the undiluted supernatant fluid before addition to a clot. The clots were then incubated for 18 hr. at 37°, after which a small volume of gelatin saline was pipetted on to each coverslip. Where the fibrin had been digested, the end-point was clearly seen by the absence of the mauve clot and the appearance of dye in the fluid. The dilution of supernatant fluid which contained 1 MLyD was thus easily estimated.

The L+ dose, defined in the usual way as the smallest amount of staphylokinase which, when mixed with one unit of antibody, results in dissolution of the clot, was then determined. The usual procedure in which serial dilutions of the test toxin are mixed with a constant unitage of standard antitoxin was reversed, serial dilutions of a standard antiserum (Wellcome Laboratories, CPP 76/63, given an arbitrary value of 100 units anti-staphylokinase/ml.) at 100 % differences being mixed with a constant amount of the staphylokinase preparation containing at least 4 MLyd. The technique was as follows: 0·01 ml. of each dilution of antiserum and 0·01 ml. of supernatant fluid containing 4 MLyD/ml. were mixed and placed on preformed fibrin clots. The preparations were incubated at 37° for 18 hr. and the results read as before. A more accurate determination of the L+ dose was then made by decreasing the differences between the dilutions of antiserum to 20 %. The amount of staphylokinase/mg. bacterial dry wt. was then calculated and expressed as L+ units of staphylokinase/mg. bacterial dry wt.

Assay of α-haemolysin

The L_H dose was determined by mixing serial dilutions of standard antitoxin at 20 % differences with a constant amount of culture supernatant fluid and adding 3 % (v/v) washed rabbit red cells, using the semi-micromethod of Gladstone & Glencross (1960). The results were related to bacterial growth and expressed as L_H/mg. bacterial dry wt.

Assay of S and F components of PV leucocidin

Woodin (1959, 1960) showed that PV leucocidin contained two components, S and F. These were estimated by the method of Gladstone & Glencross (1960) in which serial dilutions of antitoxin containing known arbitrary unitages of anti-F and anti-S at 50 % differences were mixed with a constant volume of supernatant fluid in the presence of excess of purified S or F components respectively, and placed on preparations of human leucocytes which were examined microscopically under phase contrast. The results were expressed as L+ units of F and S/mg. bacterial dry wt.

Assay of hyaluronidase

Estimations of hyaluronidase were made by a modification of the turbidity-reduction method of Pearce (1953). A standard curve was prepared by measuring the turbidity produced by different quantities of a solution of hyaluronic acid (from Dr D. Riding, Evans Biological Institute) dissolved in 0·1 M-acetate buffer (pH 6·0) containing 0·15 N-NaCl, made up to 1 ml. with 0·2 % (w/v) gelatin acetate buffer (pH 6·0) 5 min. after the addition of 5 ml. acidified protein reagent (0·1 %, w/v, bovine plasma fraction V, Armour, in 0·1 M-acetate buffer pH 4·2, adjusted to pH 3·75 with 4 N-HCl). All estimations were done in duplicate and the optical density at hyaluronidase concentrations of 50, 100, 150 and 200 μg./ml. measured before each experiment to check the slope and position of the curve.

The culture supernatant fluids were adjusted to pH 6·0 with 2 N-HCl and dilutions prepared in 0·2 % (w/v) gelatin acetate buffer (pH 6·0). To 0·5 ml. of each dilution was added 0·5 ml. of the solution of hyaluronic acid containing 400 μg. hyaluronic acid/ml. in spectrophotometer tubes. These were incubated at 37° for exactly 10 min. and then transferred to a 70° water-bath for 30 min. to inactivate hyaluronidase. After the solutions had cooled to room temperature, 5 ml. acidified protein reagent were added to each tube at 1 min. intervals, and the turbidity read after exactly 5 min. The amount of hyaluronic acid left was read from the standard curve and the amount of hyaluronidase present calculated in turbidity reducing (t.r.) units. One t.r. unit is defined as the amount of hyaluronidase which will decrease the turbidity given by 200 μg. hyaluronic acid to that given by 100 μg. hyaluronic acid. The results were expressed as t.r. units/mg. bacterial dry wt.

RESULTS

Experiments to determine the optical conditions for the production and assay of staphylokinase

Optimal time of harvesting. Cultures were incubated at 37° and 1 ml. samples taken at intervals to determine the yield of staphylokinase at different times. Graphs of growth and yield of staphylokinase against time of incubation showed that production of staphylokinase at first lagged behind growth, but then proceeded rapidly, reaching a maximum yield per ml. at about 6½ hr. for all three strains under these conditions (Fig. 1). It was therefore decided to incubate all cultures for 6½ hr. after the 10 % inoculum had been made.

Optimal time of incubation with the test fibrin clots. Six parallel series of MLyD tests were prepared and the results read after 1, 2, 4, 8, 18 and 24 hr. The longer times were used as it is known (Gerheim & Ferguson, 1949) that staphylokinase requires long incubation to activate plasminogen. After 1 or 2 hr. of incubation no lysis had occurred and as the time was increased from 4 to 18 hr., the end-point was found in progressively higher dilutions of the supernatant fluid. Incubation beyond 18 hr. gave no further change in the end-point and all further tests were therefore incubated for this time.

Variability of results. Twenty samples of the same culture supernatant fluid were tested to see whether the staphylokinase end-point was always found in the same dilution. All the tests gave an end-point with the same dilution of antiserum or with one different by only 20 %. A series of tests with different dilutions of supernatant fluid showed that a stoichiometric relationship existed between supernatant fluid dilution and antiserum dilution (Table 1).

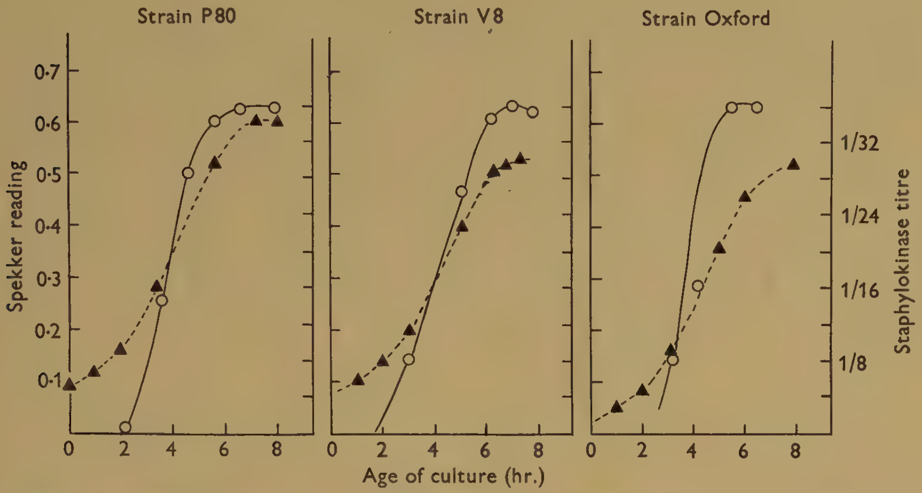

Fig. 1. Relationship between time of incubation of cultures and growth and yield of staphylokinase. ▲—▲, growth; ○—○, yield of staphylokinase.

Effect of temperature on the production of staphylokinase

Cultures were incubated at 35°, 37°, 39°, 41° and 43° and the yields of staphylokinase assayed. Occasionally growth at 43° was very low; in such cases, the cultures were discarded and new ones grown. In all other cases, the growth obtained was of the same order, i.e. about 5–6 mg. bacterial dry wt./ml. In every test, cultures at 37° were included as controls.

Table 1. *α-Haemolysin activity: relationship between dilution of supernatant fluids of staphylococcal cultures and dilutions of antiserum*

Reciprocals of dilutions of supernatant fluids	Reciprocals of dilutions of antiserum							
	32	64	128	256	512	1024	2048	4096
	lysis (+) or no lysis (−) of clot							
2	−	−	+	+	+	+	+	+
4	−	−	−	+	+	+	+	+
8	−	−	−	−	+	+	+	+
16	−	−	−	−	−	−	+	+
32	−	−	−	−	−	−	+	+

The amount of staphylokinase/mg. bacterial dry wt. detected at the different temperatures was greatest at 35° and smallest at 43° (Fig 2). When the logarithm of the amount of staphylokinase (y) was plotted against temperature (x) the regression of y on x was linear. All three strains showed the same slope, i.e. $-0{\cdot}0702$ ($\pm 0{\cdot}0064$) but the positions of the lines differed. The curves for strains V8 and

Oxford were identical, while that for strain P80 was significantly lower. The coefficient 0·0702 implies that the amount of staphylokinase produced decreased by 18 % for each degree rise in temperature.

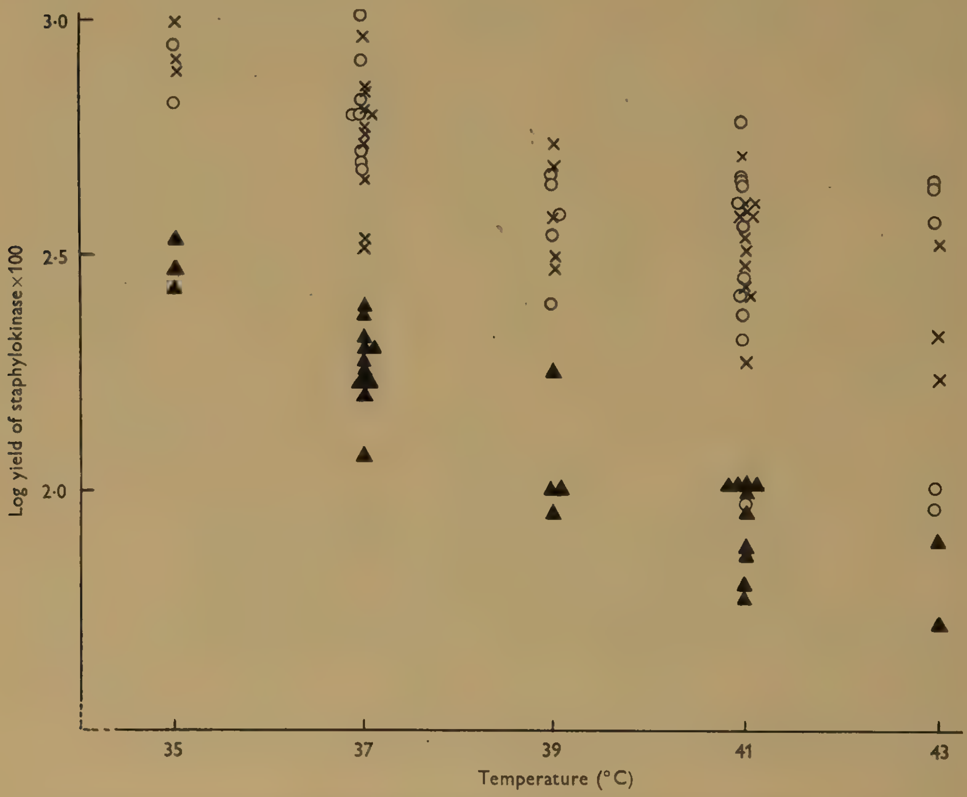

Fig. 2. Yields of staphylokinase produced by staphylococcal strains P80, V8 and Oxford at temperatures ranging from 35° to 43°. ▲, P80; ○, V8; ×, Oxford.

Several samples of supernatant fluid from cultures of all three strains at 37° containing a known amount of staphylokinase were kept at 43° for 6½ hr. and then re-tested. The amount of staphylokinase was unchanged and it was concluded that the lower yields obtained at the higher temperatures were not due to destruction of staphylokinase.

Effect of temperature on the production of α-haemolysin

Cultures of the staphylococci grown at temperatures ranging from 37° to 41° were tested for α-haemolysin content. The values obtained did not differ significantly at the different temperatures.

Effect of temperature on the production of F and S components of leucocidin

Supernatant fluids tested for F and S components of leucocidin showed no differences in the amounts produced at the different temperatures.

Effect of temperature on the production of hyaluronidase

Two temperatures, 37° and 41°, were selected for these tests; a comparison of the amounts of hyaluronidase produced at these temperatures is shown in Table 2. The supernatant fluids from cultures incubated at 37° contained about 5–8 times as much hyaluronidase as those incubated at 41°. When supernatant fluids from cultures incubated at 37° were kept at 41° for 14 hr., the results obtained were unchanged, showing the original difference to be due to decreased production of hyaluronidase.

Table 2. *Production of hyaluronidase at* 37° *and* 41°

Strain	t.r.u. per mg. at 37°	t.r.u. per mg. at 41°
P80	8·0	1·2
	6·0	< 1·0
	5·0	< 1·0
	5·5	1·2
	7·3	1·4
V8	10·7	2·4
	8·9	2·1
	7·2	1·6
	> 10·0	2·45
	6·7	1·5
Oxford	30·0	4·8
	27·2	3·4
	> 13·0	4·4
	19·0	3·1
	17·0	2·5

DISCUSSION

The experiments described show that for these three strains of coagulase-positive staphylococci, growth at a temperature higher than 35° resulted in a progressive and marked diminution in the yields of staphylokinase and hyaluronidase, while total mass of growth, α-haemolysin and PV leucocidin remained unaltered. If it be permissible to apply these results to conditions of staphylococcal sepsis *in vivo*, then a rise in body temperature of this order in fever might be held to exert a suppressive effect upon staphylokinase and hyaluronidase production in the animal tissues. Even so, the role of these and other extracellular substances produced by staphylococci in relation to invasiveness and virulence is obscure. Hyaluronidase production seems unrelated to virulence for any given strain (see Bøe, 1944). The use of staphylokinase and coagulase production as indicators of staphylococcal virulence has been challenged. Kapral & Isabel (1960) produced mutants which had lost the power to produce staphylokinase (both bound and soluble), yet these strains were as virulent for rabbits as was the parent strain. Another mutant, which retained its ability to produce coagulase, almost completely lost its invasiveness and virulence. Virulence, as many observers have suggested, is probably a complex phenomenon in which many products of the staphylococcus, some as yet unidentified, may be involved.

I wish to thank Dr G. P. Gladstone for guidance and help in this work; Mr J. Scott for the statistical analysis of the results; Drs R. A. Kekwick, D. Riding and

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Experimental Conditions for Nitrate Reduction by Certain Strains of the Genus *Lactobacillus*

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SUMMARY

Costilow & Humphreys's (1955) observation that certain strains of *Lactobacillus plantarum* reduced nitrates under certain conditions was confirmed. Two strains of *L. fermenti* also reduced nitrates. In static culture, agar and anaerobiosis were not essential for nitrate reduction, contrary to speculations in the literature. Nitrate reduction was possible only in media with restricted carbohydrate and with the pH value maintained at a relatively high value within the activity range of nitrate reductases. For good growth, lactobacilli for the nitrate test have been customarily grown in media with high carbohydrate content, with consequent low final pH values. This seems to be the essential reason why the genus *Lactobacillus* had previously been defined as unexceptionally nitratase-negative.

INTRODUCTION

Until recent years the genus *Lactobacillus* has been described as unable to reduce nitrates (*Bergey's Manual*, 1948; Rogosa *et al.* 1953). However, Costilow & Humphreys (1955) reported that 18 of 38 strains of *Lactobacillus plantarum* reduced nitrates. This result was achieved through the use of the BBL indole-nitrite medium (Baltimore Biological Laboratory, Inc.) which has an initial pH value of 7·2 and a composition of 2 % (w/v) Trypticase, 0·2 % (w/v) Na_2HPO_4, and 0·1 % (w/v) each of glucose, KNO_3 and agar. Negative results, consistent with earlier general experience, were obtained with broth media, such as the BBL indole-nitrite medium minus agar, or Difco nitrate broth. To explain this, Costilow & Humphreys (1955) reasoned that agar would tend to decrease the oxygen tension of the medium and stated: 'It is obvious that the oxygen tension of the medium was the most important factor in nitrate reduction....' From inspection of the formula of the BBL indole-nitrite medium it is evident that it contains very little fermentable carbohydrate (0·1 %, w/v) and is very highly buffered. Also the initial pH, 7·2, is high. This suggests that nitrate reduction may be a function of pH value and that the nitrate reductase enzymes may have a relatively high and narrow pH activity range. For example, Woods (1938) found with *Clostridium welchii* that, when the reduction rate at pH 6·8 was taken as 100 %, it was 50 % at pH 6·4 and only 10 % at pH 6·1 after 25 min. After 35 min., the reduction rate decreased further at pH 6·5 to only 10 %. Similar results were obtained with a strain of

Escherichia coli. Nason & Evans (1955) studied a purified enzyme from *Neurospora crassa* which catalysed the reaction $TPNH + H^+ + NO_3^- \rightarrow TPN^+ + NO_2^- + H_2O$ and the enzyme exhibited a sharp optimum for activity at pH 7·0 in phosphate buffer. Zucker & Nason (1955) found maximum activity between pH 8·0 and 9·0 for the intermediate enzyme, hydroxylamine reductase, in denitrification by *N. crassa.* Najjar (1955) obtained similar high pH optima with *Pseudomonas stutzeri* and *Bacillus subtilis* for the enzymic conversion of NO_2^- and NO to N_2. Because of the importance of nitrate reduction as a taxonomic criterion, a wide range of *Lactobacillus* species, and certain streptococci and pediococci, were tested in experiments designed to discover the appropriate conditions for nitrate reduction.

METHODS

The strains of lactobacilli were representative of 13 species in the collection of Dr M. Elisabeth Sharpe (National Institute for Research in Dairying, NIRD, University of Reading), obtained through original isolations by Dr Sharpe or other workers, and from such culture collections as the American Type Culture Collection (ATCC), the (British) National Collection of Type Cultures (NCTC), the (British) National Collection of Industrial Bacteria (NCIB) and the (British) National Collection of Dairy Organisms (NCDO maintained at NIRD). Each strain had been carefully studied by numerous tests (Rogosa *et al.* 1953; Rogosa & Sharpe, 1959) including serological procedures (Sharpe, 1955) wherever specific group antisera could be prepared.

There were strains of *Lactobacillus plantarum.* The origins of the following 7 are of interest: *AR1* NIRD 17/5; *P4*, NCTC 6376 from ATCC 8014; *P16*, ATCC 8014; *AR5*, *L. arabinosus* V743 Tittsler; *P30*, V322 Tittsler; *P7*, NIRD 1–4; *P8*, NIRD 1–8. Strains *AR1*, *P4*, *P16* and *AR5* are identical and are really only a single strain designated *L. arabinosus* 17/5 by Fred, Peterson & Anderson (1921), but ascribed different numbers in different culture collections.

Five strains of *Lactobacillus fermenti* were tested. Strain *F1* is identical with ATCC 9338 and NCTC 6991, and strain *F4* is NCTC 7230.

The remaining strains were distributed numerically as follows: *Lactobacillus acidophilus*, 5; *L. brevis*, 10; *L. buchneri*, 1; *L. bulgaricus*, 9; *L. casei*, 14; *L. cellobiosus*, 2; *L. delbrueckii*, 2; *L. helveticus*, 1; *L. jugurti*, 3; *L. lactis*, 11; *L. leichmannii*, 2; *L. salivarius*, 4.

In addition, one strain each of the following group D streptococci was tested: *Streptococcus bovis*, *S. durans*, *S. faecalis* var. *liquefaciens*, *S. faecalis* and *S. faecium.* Among group N streptococci were two strains of *S. cremoris*, one of *S. lactis* and two of *S. lactis* var. *diacetilacticus.* Three strains of *Pediococcus cerevisiae*, including ATCC 8081, were also tested.

The media used were: (1) the BBL indole-nitrite medium; (2) Difco nitrate broth; (3) various modifications of these such as the addition of agar, yeast extract, increased buffering with Na_2HPO_4, and changes in the concentration of glucose. The conditions for each experiment will be described in the tabulation of results.

Cultures were inoculated with one drop from good growth in MRS broth (de Man, Rogosa & Sharpe, 1960) and incubated for 6–7 days at 37° except for *Lactobacil-*

lus plantarum, *L. brevis*, *L. casei*, the streptococci and the pediococci which were incubated at 30°.

When anaerobiosis was desired it was obtained by using McIntosh & Fildes jars filled with either H_2 or a 90 % H_2 + 10 % (v/v) CO_2 mixture, and combining residual traces of O_2 with H_2 by using the electrically heated catalyst.

The reagents for the detection of nitrite were: solution 1 containing 2 g. sulphanilic acid in 250 ml. 5N-acetic acid; solution 2 containing 1·5 ml. dimethyl-α-naphthylamine in 250 ml. 5N-acetic acid. The latter reagent was recommended by Wallace & Neave (1927) and Tittsler (1930) as superior to the α-naphthylamine reagent because the colour develops instantaneously, is more intense, and does not fade in a reasonable period of time. For the nitrite test, one drop of culture was deposited in a white porcelain spot plate followed by one drop each of solutions 1 and 2. The pink to red colour in positive tests developed at once and was unequivocal. The spot plate technique was often superior to tests made directly in the tubes. Some media contain substances (thioglycollate, for example) which inhibit colour development, but with the quantities of culture and reagents described for the spot plate test, colour development was uninhibited. Negative controls on the media and a positive control with *Micrococcus aureus*, phage type 80, were included routinely. All negative tests were confirmed by reducing residual nitrate to nitrite with Zn dust on the spot plate and testing for nitrite as described. Zn reductions in the tubes often gave ambiguous and false results.

RESULTS

The only species in which some strains reduced nitrates were *Lactobacillus plantarum* (7 of 12) and *L. fermenti* (2 of 5 tested). All other species of lactobacilli, and the streptococci and pediococci examined, were unable to reduce nitrates under any of the experimental conditions. Thus, Costilow & Humphreys's (1955) observation that certain strains of *L. plantarum* can reduce nitrates under appropriate conditions was confirmed.

Some typical data from experiments concerning the role of agar, yeast extract, initial pH and anaerobiosis in nitrate reduction by *Lactobacillus plantarum*, are presented in Table 1. When Difco nitrate broth (DNB) or DNB supplemented with agar and yeast extract was used at an initial pH 6·4 (a pH value favourable for growth) all strains repeatedly failed to reduce nitrates aerobically or anaerobically and the pH value decreased sharply to a final value of 3·4. At the relatively unfavourable value pH 7·4, however, the cultures reduced nitrates, the pH decrease was markedly less, and the final pH value was about 6·4, i.e. 3 units less than the decrease obtained when the initial value was pH 6·4.

In BBL indole-nitrite (IN) medium nitrate reduction was sometimes suppressed under anaerobic conditions, as illustrated by the data for strain P30. In IN medium supplemented with Difco yeast extract (YE) nitrate reduction was also occasionally absent or weak. From observation of growth it was obvious that these weak or negative responses were related to total growth and that growth in IN medium + YE was even poorer than in the relatively poor IN medium. Also, it is clear from Table 1 that nitrate reduction, both aerobically and anaerobically, took place in the absence of agar, which was irrelevent to the process. Yeast extract in broth media,

Table 1. *Effect of composition of culture medium, anaerobiosis, and* pH *value on nitrate reduction by* Lactobacillus plantarum

	Uninoculated control				Strain P30				Strain P4			
	Aerobic		Anaerobic†		Aerobic		Anaerobic		Aerobic		Anaerobic	
Experimental medium	NO_2*	pH	NO_2	pH	NO_2	pH	NO_2	pH	NO_2	pH	NO_2	pH
Difco nitrate broth (DNB)	–	6·4	–	5·9	–	3·4	–	3·4	–	3·4	–	3·4
DNB+0·1 % agar	–	6·4	–	6·0	–	3·4	–	3·4	–	3·4	–	3·4
DNB+0·1 % agar+0·3 % Difco yeast extract (YE)	–	6·4	–	6·0	–	3·4	–	3·4	–	3·4	–	3·4
DNB+0·1 % agar+0·3 % Difco yeast extract (YE)	–	7·4	–	6·0	+	6·1	–	6·0	+	6·4	+	5·7
BBL indole-nitrite (IN) medium	–	7·0	–	6·9	+	6·4	–	6·4	+	6·4	+	6·4
IN medium+0·3 % YE	–	7·1	–	6·9	–	6·3	–	6·4	+	6·4	sl	6·4
IN medium with agar omitted (IN broth)	–	7·1	–	6·7	+	5·7	–	5·7	+	5·8	tr	5·8
IN broth+0·3 % YE	–	6·9	–	6·7	+	5·7	–	5·8	+	5·8	–	5·8
IN broth+0·3 % YE+0·9 % glucose	–	7·0	–	6·7	–	5·5	–	4·9	–	5·6	–	5·0

* NO_2 = nitrate reduction; sl and tr = slight and trace reduction, respectively; – = negative and + = positive.
† Anaerobic conditions 90 % (v/v) H_2+10 % CO_2 (v/v).

Table 2. *Relation between fermentable carbohydrate, final* pH *value, and nitrate reduction by* Lactobacillus plantarum, *under aerobic and anaerobic conditions*

	AER*		ANH_2		$ANH+CO_2$		AER		ANH_2		ANH_2+CO_2	
Medium	NO_2†	pH	NO_2	pH	NO_2	pH	NO_2	pH	NO_2	pH	NO_2	pH
	Uninoculated control						Strain P30					
BBL indole-nitrite (IN) medium	–	7·3	–	7·3	–	6·6	+	6·6	+	6·6	–	6·3
IN medium+0·1 % glucose	–	7·3	–	7·4	–	6·6	–	5·8	tr	6·0	–	5·7
IN medium+0·3 % glucose	–	7·1	–	7·2	–	6·5	–	4·9	–	4·9	–	4·7
IN medium+0·5 % glucose	–	7·1	–	7·2	–	6·6	–	4·7	–	4·6	–	4·2
IN medium without agar	–	7·3	–	7·4	–	6·6	+	6·8	+	6·6	–	6·4

	AER		ANH_2		ANH_2+CO_2		AER		ANH_2		ANH_2+CO_2	
	NO_2	pH	NO_2	pH	NO_2	pH	NO_2	pH	NO_2	pH	NO_2	pH
Medium	Strain P4						Strain AR5					
BBL indole-nitrite (IN) medium	+	6·8	+	6·6	+	6·4	+	6·8	+	6·8	+	6·2
IN medium+0·1 % glucose	+	5·9	+	5·9	+	5·8	+	6·0	–	6·0	sl	5·7
IN medium+0·2 % glucose	–	4·8	–	4·9	–	4·8	+	5·0	–	5·0	–	4·9
IN medium+0·5 % glucose	–	4·5	–	4·4	–	4·4	–	4·5	–	4·5	–	4·3
IN medium without agar	+	6·8	+	6·7	+	6·6	+	6·9	+	6·8	+	6·5

* AER = aerobic incubation; ANH_2 = anaerobically in 100 % H_2; ANH_2+CO_2 = anaerobically in 90 % (v/v) H_2+10 % CO_2 (v/v).
† NO_2 = nitrate reduction; – = negative and + = positive; sl and tr = slight and trace reduction, respectively.

contrary to the experience with agar-containing media, did not inhibit nitrate reduction. What is most germane to this discussion is that cultures in media such as IN medium without agar + YE supplemented with conventional quantities of glucose (0·9 %, w/v), had relatively low terminal pH values and also did not then reduce nitrates.

The above results strongly indicate that induced anaerobic conditions, yeast extract, and agar are not essential for nitrate reduction by these strains. Rather, it appears that the pH value, fermentable carbohydrate, and buffer capacity are the principal factors which effect nitrate reduction. Table 2 presents typical data which confirm this hypothesis. Anaerobiosis previously was achieved with 90 % (v/v) H_2 + 10 % (v/v) CO_2. Since CO_2 may decrease the pH value of the medium, anaerobiosis in further experiments was also maintained by an atmosphere of 100 % H_2. As little as 0·1 % (w/v) added glucose inhibited nitrate reduction by *Lactobacillus plantarum* P30. Nitrate reduction by this strain seemed most sensitive to slight acidity from the fermentation of glucose, but all nitrate positive strains in IN medium were, with one exception, unable to reduce nitrates when 0·3 % (w/v) glucose was added and the pH value consequently decreased to 5·0 or less. With 0·5 % (w/v) glucose, nitrate reduction was never observed. Again, positive reactions were generally fewer or less intense in anaerobic as compared with aerobic cultures, and it is quite clear that agar had no enhancing effect. These repeated results with agar do not agree with those of Costilow & Humphreys (1955). In experiments which repeated their experimental protocol exactly as described, but in which the pH values of comparable broth and agar media were adjusted electrometrically exactly to the same initial value of 7·1, there was no difference in nitrate reduction in broth or agar containing media.

The strain of *Micrococcus aureus*, phage type 80, which was used as a positive control, reduced nitrate to nitrite at pH 7·0. But at pH 7·5 or above the organism consistently also reduced nitrite completely, with accumulation of N_2. In this case it would appear that the nitrite reductase enzyme had an even higher pH activity range than the nitrate reductase.

DISCUSSION

Clarke (1959) emphasized the nitrate reducing property of some anaerobic bifid organisms named *Lactobacillus bifidus*. In the judgement of the writer and of Orla-Jensen, Orla-Jensen & Winther (1936), Rogosa & Sharpe (1959), and Sundman, Bjorksten & Gyllenberg (1959) who described these organisms as 'organisms previously incorrectly designated *Lactobacillus bifidus*', these organisms should not be properly within the genus *Lactobacillus* but rather be allocated elsewhere. A more extensive discussion is reserved for the future.

The plausible argument that anaerobic technique is essential for nitrate reduction in static culture is not new. ZoBell (1932), in a study of nitrate reduction by *Brucella* spp., advocated the use of a medium made semisolid with 0·3 % (w/v) agar, which he thought would lower the O/R potential and thus encourage nitrate reduction. The basal medium was a poor one containing only 0·2 % (w/v) peptone, 0·1 % (w/v) beef extract, and 0·3 % NaCl (w/v) as nutrients. He observed markedly improved growth in the semisolid medium. In the absence of comparative aerobic and anaerobic results in broth media it is just as reasonable to assume that the agar was exerting nutritional effects or was adsorbing toxic materials.

It has already been shown in the present experiments that agar and anaerobic cultivation are unessential for nitrate reduction by *Lactobacillus plantarum*. The statement by Costilow & Humphreys (1955) 'that oxygen tension is a very important factor in the ability of bacteria to reduce nitrates' and their remarks concerning Sacks & Barker (1949) can be misunderstood. Sacks & Barker (1949) said: 'Weissenberg (1897) tested the ability of three denitrifying bacteria to reduce nitrate and nitrite in shallow layers of medium exposed to air and in the complete lack of oxygen. Weissenberg found that complete denitrification occurred in the anaerobic cultures, *whereas aerobically nitrate was reduced only as far as nitrite*.' (Italics mine.) Sacks & Barker proceeded to test the effect of different partial pressures of oxygen on denitrification by *Pseudomonas denitrificans*, using special precautions to maintain equilibrium between the atmosphere and the culture medium. This was done in manometric experiments by shaking the flasks at the rapid rate of 150 oscillations/min. Under these conditions both nitrate and nitrite reduction were inhibited.... 'but even in a solution fully saturated with air the rate of nitrate reduction is 29 per cent of the anaerobic rate'. The influence of oxygen tension on the formation of nitrate- and nitrite-reducing enzyme systems during growth was determined in experiments in which the bacteria were grown anaerobically 'and aerobically while being vigorously aerated with 1·0, 5·0, or 20·6 per cent oxygen in nitrogen'. It was found that: 'The formation of *nitrite*-reducing enzymes is decreased 29 per cent by 1 per cent oxygen and is completely prevented by oxygen at a level of 5 per cent or higher. The lowest oxygen level capable of preventing the formation of *nitrite*-reducing enzymes may be considerably below 5 per cent; no data are available in the range between 1 and 5 per cent oxygen. The formation of *nitrate*-reducing enzymes is much less sensitive to oxygen during growth; 1 per cent oxygen causes no detectable inhibition and even saturation of the culture medium with air does not completely prevent the formation of such enzymes.' (Italics mine.) Furthermore, at an oxygen tension of 5 % when nitrite reduction was nil, nitrate reduction was still 65 % of the anaerobic reaction.

The usual conditions for the nitrate-reduction tests are static cultures, not 'aerobically while being vigorously aerated'. Also, negative oxidation-reduction potentials are reached by lactic acid bacteria in carbohydrate media during early 'aerobic' growth (Frazier & Whittier, 1931). It seems highly probable that the deleterious effect of oxygen on nitrate reduction has not been clearly differentiated from its effect on nitrite reduction and that an argument has been applied from the inapplicable case of denitrifying bacteria which reduce nitrates to nitrites and then reduce nitrites to other products. But in the routine test for nitrate-reduction the reagents test for nitrite accumulation. Even with denitrifying bacteria which also reduce nitrite, the disappearance of nitrite and residual nitrate indicates a positive reaction.

Some years ago the writer's laboratory tested hundreds of lactobacilli for nitrate reduction in anaerobic culture with completely negative results. Included were many strains of *Lactobacillus plantarum*, among which were some that were positive when tested later by Costilow & Humphreys (1955). These have also been found to reduce nitrate in the present work. Thus, the argument which involves decreased oxygen tensions was not appealing in itself as an explanation for previous negative results. We have seen that the reaction of the medium is a critical factor in the reduction of nitrates. Generally, nitrate media for denitrifying bacteria contain extremely little added carbohydrate or more often none at all. It is not difficult to see now that with the acidogenic lactobacilli relatively small concentrations of fermentable carbohydrate may inhibit or prevent nitrate reduction because of pH changes. It has previously been customary to culture lactobacilli for the nitrate test in media containing enough carbohydrate for good growth. With the accumulation of acidic fermentation products the pH value, even in early growth, shifts beyond the activity range of nitrate reductases. This seems the highly probable reason why the genus *Lactobacillus* has previously been described as unexceptionally nitrate-negative.

It is not clear why only certain strains of *Lactobacillus plantarum* and *L. fermenti* were nitrate-positive. There were no obvious correlations between this and any other properties of the organisms. Most species grew poorly in the media tried. Attempts to improve growth by the use of massive inocula of washed organisms or to induce adaptation, and changes in the buffer content of media, have not yet changed the nitrate-negative character of any organisms.

I gratefully acknowledge the hospitality of the Director and staff of the National Institute for Research in Dairying, University of Reading, England, where most of this work was done. Particularly I acknowledge the kind help of Dr M. Elisabeth Sharpe who provided me with laboratory facilities, technical aid and many cultures.

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ADDENDUM

Clarke (1959) reported nitrate reduction by *Lactobacillus brevis* NCTC 2797. Dr M. Elisabeth Sharpe of the NIRD has confirmed that this strain reduces nitrates under appropriate conditions as described in this paper; but she has found that in fact this strain is not *L. brevis* but is *L. fermenti* as confirmed by nutritional and serological tests and growth temperatures.

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Purification and Properties of Neuraminidase from *Vibrio cholerae*

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SUMMARY

A method is described for the purification of neuraminidase from culture fluids of *Vibrio cholerae*. Five steps are involved: fractionation with methanol, adsorption to and elution from human red cells, fractionation with ammonium sulphate, chromatography on columns of hydroxyl apatite, crystallization. From 35 l. of culture filtrate, an average yield of 21 % of the original enzyme activity was obtained as crystals. The degree of purification was about 5000 fold. Purified neuraminidase possessed $12{\cdot}6 \times 10^6$ units of biological activity/mg. dry weight, and gave a value for $E^{1\%}_{280\,\mathrm{m}\mu}$ of 8·96, measured at 0·025 % (w/v). Enzyme activity was stimulated by calcium ions and inhibited by ethylenediaminetetra-acetate. In the presence of 0·001 M-$CaCl_2$, neuraminidase showed maximum activity at pH 5·6. With sialyl lactose as substrate, a value of $1{\cdot}2 \times 10^{-3}$ M was found for the Michaelis constant. At an enzyme concentration of 0·16 μg./ml., $V_{\mathrm{max.}}$ was 0·021 μM *N*-acetylneuraminic acid/min./ml. The enzyme was stable when dried from the frozen state and stored under vacuum at 0°. A suspension of crystals in water also retained activity when stored at 0°. Solutions of crystalline neuraminidase showed a small increase in activity when stored at 0° for several weeks. This effect was greatest at pH 6·7 and 8·5 but was barely detectable at pH 4·6. At pH 5·6 or 6·7, the enzyme lost about 20 % of its activity over a period of 2 hr. at 37° (concentration = 15 μg./ml.) No proteolytic activity nor *N*-acetylneuraminic acid aldolase activity was detected in the crystalline preparation.

INTRODUCTION

The ability of culture fluids of *Vibrio cholerae* to render human red cells inagglutinable by influenza virus was first noticed by Burnet, McCrea & Stone (1946) who described the active principle as the receptor-destroying enzyme (RDE) of *V. cholerae*. Gottschalk & Lind (1949) early provided evidence about the chemical activity of RDE but the enzyme was not characterized as a glycosidase until 1956 (Gottschalk, 1956; Heimer & Meyer, 1956). The enzyme has been termed sialidase (Heimer & Meyer, 1956) or neuraminidase (Gottschalk 1957) and its action defined as 'the hydrolytic cleavage of the glycosidic bond joining the keto group of *N*-acetylneuraminic acid to D-galactosamine' (Gottschalk 1957).

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Early work on the purification of the enzyme (Ada & French, 1950) was hampered by the complex nature of the culture medium and the low enzyme titre obtained. These difficulties were overcome when it was found that addition of *N*-acetylneuraminic acid or certain derivatives to a simple medium greatly stimulated enzyme production (Ada & French, 1957, 1959*a*; French & Ada, 1959). A dialysate of bovine colostrum enriched with glycerol and peptone was found to be a suitable medium for enzyme production (Ada & French, 1957). Following growth in this medium and subsequent heating to kill the organisms, filtration yielded a crude extract from which it was possible to purify the enzyme (Ada & French, 1959*b*). The present paper presents details of the purification procedure and some properties of the crystalline enzyme. The succeeding paper (Pye & Curtain, 1961) reports some physical properties of the enzyme.

METHODS

Peptone. Evans bacteriological peptone or Difco proteose peptone no. 1 was used.

Glycerol. A.R. quality.

Peptone water. A solution of glycerol (0·5 %, v/v) and peptone (1 %, w/v) adjusted to pH 7·4 with NaOH and sterilized by autoclaving (15 lb./sq.in.; 20 min.)

Methanol. A preparation labelled 'acetone-free' was found to be satisfactory.

Anti-frothing reagent. Undecanol (3 %, v/v) in peanut oil; sterilized by heating for 1 hr. at 180° on 3 successive days.

EDTA. Ethylenediaminetetra-acetic acid solution, adjusted to pH 7·4 with NaOH.

Hydroxyl apatite. Prepared according to Tiselius, Hjerten & Levin (1956). This material could be stored at 0–4° for periods of at least 1 month.

Sialyl lactose. The method of preparation was described by French & Ada (1959).

Saline. An aqueous solution of NaCl (0·15 M).

Bovine plasma albumin. Fractionation V prepared by the Armour Laboratories.

Phosphate buffers. Phosphate buffers (pH 6·8) were prepared by dilution of M stock solution (0·5 M-NaH_2PO_4; 0·5 M-Na_2HPO_4).

Tris-maleate buffers. These were prepared by mixing 0·1 M 2-amino-2-hydroxymethylpropane-1:3-diol (tris) and 0·1 M-maleic acid solutions.

Estimation of protein concentration. This was generally determined by the method of Lowry, Rosebrough, Farr & Randall (1951). On some occasions, it was assessed from optical density measurements at 280 mμ. In certain kinetic studies, a sample of the enzyme solution was dried (100°) and weighed.

Estimation of N-*acetylneuraminic acid* (N-*ANA*). Total *N*-ANA was estimated by the direct Erhlich reaction (Werner & Odin, 1952). Free *N*-ANA was estimated by the thiobarbituric acid method (Warren, 1959).

Drying of neuraminidase. Samples of the purified enzyme were dried from the frozen state *in vacuo* in an apparatus described elsewhere (Holden, 1958). We wish to thank Mr H. F. Holden for this courtesy.

Titration of neuraminidase activity

(*a*) *Biological method.* The method used and definition of a unit of enzyme activity has been described elsewhere (French & Ada, 1959).

(*b*) *Chemical method.* The substrate solution was constituted as follows: sialyl lactose solution (10 %, w/v), 0·8 ml.; $CaCl_2$ solution (0·01 M), 1·5 ml.; tris + maleate buffer (pH 5·6) containing 0·05 % (w/v) bovine plasma albumin, 12·7 ml. Samples (0·35 ml.) of this were brought to 37·1° and 0·05 ml. of enzyme solution added. At appropriate intervals (usually 10 min.), 0·1 ml. samples were pipetted into tubes containing 0·1 ml. water, 0·1 ml. of the periodate reagent (Warren, 1959) was added immediately and the amount of liberated *N*-ANA determined. In all experiments, the rate of release of *N*-ANA at zero time was estimated by extrapolation.

Titration of proteolytic activity

Casein substrate. Defatted casein (0·5 g.) was suspended in water and 0·1 N-NaOH added cautiously until the protein just dissolved (final volume = 100 ml.). This solution was dialysed for 16 hr. at 0° against veronal + acetate buffer.

Buffer. 25 ml. 0·04 M-sodium barbiturate, 25 ml. 0·04 M-sodium acetate, adjusted to pH 6·5 with N-HCl. Volume brought to 100 ml.

Trypsin. A crystalline preparation was dissolved in buffer, dilutions made in buffer and used immediately.

Test. Enzyme solution (0·1 ml.) was added to 1 ml. samples of the substrate solution, the solutions mixed and then incubated for 1 hr. at 37°. After cooling in ice water, 0·25 ml. of 40 % (w/v) trichloroacetic acid was added, the solutions mixed, kept at 0° for 15 min. and then centrifuged (2000 *g*; 15 min. 0°). The absorption at 280 mμ of the clear supernatant liquid was read in 0·5 cm. optical cells.

Titration of N-*ANA-aldolase activity*

N-*ANA.* A crystalline preparation derived from sialyl lactose (French & Ada, 1959).

N-Acetylmannosamine. A crystalline sample kindly provided by Dr S. Roseman.

Test. To 0·2 ml. of *N*-ANA solution (1·7 %, w/v) was added 0·3 ml. of phosphate buffer (0·01 M; pH 6·8) or neuraminidase dissolved in 0·3 ml. buffer. One drop of toluene was added, and after removal of 0·15 ml. as the zero time sample, the tubes were stoppered and incubated at 37° for 24 hr. The *N*-acetylhexosamine content of the zero time and 24 hr. samples was estimated by the method of Reissig, Strominger & Leloir (1955), with *N*-acetylmannosamine as standard.

Production of neuraminidase

Organism. The properties, cultivation and storage of the 4Z strain of *Vibrio cholerae* were described previously (French & Ada, 1959). Twenty-four hr. before bulk growth of the organisms, nutrient broth (100 ml.) was inoculated from an agar slope culture and incubated (6 hr., 37°). The whole of this culture was used as inoculum for 1 l. peptone water which was then incubated for 16 hr. at 37°. The peptone water culture was used as inoculum for the bulk medium (see below).

Preparation of medium. Bovine colostrum (about 6 l.; first milking after parturition) was dialysed (Visking 27/32 tubing) against 35 l. water previously saturated with chloroform. Dialysis was carried out at 0–4° for 8–14 days in a stainless steel can with a closely fitting lid, the can being briefly shaken each day. The colostrum was then discarded, the clear diffusate warmed to about 45° and the chloroform removed by aeration with sterile air (2–3 hr.). Glycerol was added to a final concentration of 0·75 % (v/v). Peptone (360 g.) was dissolved in 1 l. of water, the pH adjusted to about 7 and insoluble material removed by centrifugation. The clear supernatant liquid was added to the colostrum dialysate and adjusted to pH 7·3 with 20M-NaOH. The medium was filtered (0–4°) through sterilizing pads into sterile containers.

Growth of organism. The medium was transferred to a sterile stainless-steel can and warmed to and maintained at 37°. The can was fitted with a transparent lid through which the shaft of a stirrer and an aeration tube passed. The latter was placed so that sterile air emerged under the blades of the stirrer. The medium was inoculated with the peptone water culture of *Vibrio cholerae* (see above), the stirrer started and air passed through the medium at about 150 l./hr. Frothing was controlled by the addition of the anti-frothing preparation. Incubation was continued for about 10 hr.

Destruction of organisms and preparation of crude extract. Dry $CaCl_2$ was added to the culture to give a final concentration of 0·5 % (w/v). The organisms usually aggregated during this procedure. After adjustment to pH 6·0 with 10 N-HCl, the mixture was brought to 56°, maintained at this temperature for 30 min. to kill the organisms, and then cooled (16 hr., 0–4°). When the clumped organisms settled out, the clear supernatant liquid was obtained by decantation (the sedimented organisms amounted to about 1 l. in volume). Otherwise the organisms were removed by filtration through a pad of filter-aid. For ease of handling, the cleared liquid (supernatant liquid or filtrate) was divided into two portions for the next treatment; one part was stored at 0–4° in the presence of toluene for 2–3 days and the other part processed immediately.

Purification procedure

Methanol fractionation of crude extract. All procedures in this step were carried out at 0–2°. The cleared liquid (above) was adjusted to pH 5·0 with 10 N-HCl. Methanol, precooled to about −30°, was added during a period of about 5 hr. until the concentration reached 60 % (v/v). The resultant precipitate was allowed to settle during 2 days. Most of the clear supernatant liquid was decanted and discarded. The precipitate was recovered from the rest by centrifugation (2000 *g*; 30 min.). Cold water was added to the packed precipitate to give a slush which was then dialysed against several changes of distilled water to remove methanol (2–3 days). The suspension was centrifuged (2000 *g*; 30 min.) and the sediment re-extracted once with an equal volume of water. The supernatant liquids were pooled and called 'neuraminidase concentrate'.

Preparation of human red cell eluate. Human blood was collected into Rous and Turner fluid and the cells washed three times with saline. Unless cells were used on the day collected, a considerable degree of haemolysis occurred in this step and increased the difficulty of later purification. The washed red cells were distributed into two equal portions of 200 ml., each of which was distributed into centrifuge cups and held at 0–4°. To the 'neuraminidase concentrate' (0–4°) was added NaCl and $CaCl_2$ to give final concentrations of 0·85 % and 0·1 % (w/v), respectively. N-NaOH was added cautiously until the pH value reached 8·5. The slight precipitate which formed was immediately removed by centrifugation (2000 *g*; 10 min.;

0–4°). The clear supernatant liquid was then added to one portion of red cells, stirred for 1 min. and then centrifuged (2000 *g*; 3 min.; 0–4°). The supernatant liquid was removed and immediately added to the other portion of red cells. Mixing and centrifugation was again carried out as above. The extracted fluid (supernatant liquid) was discarded, the red cells pooled and suspended in 400 ml. saline containing 0·01 M-EDTA and kept at about 20° for 10 min., with occasional gentle stirring. The suspension was centrifuged (2000 *g*; 10 min.; 20°) and the supernatant liquid removed. The red cells were re-extracted as above with another 200 ml. of EDTA saline. The supernatant liquids were pooled and re-centrifuged (2000 *g*.; 20 min.; 0–4°), yielding a clear slightly pigmented supernatant liquid (red cell eluate).

Ammonium sulphate fractionation. An equal volume of aqueous saturated $(NH_4)_2SO_4$ (pH 6·5) was immediately added to the red cell eluate. After standing for 16 hr. at 0–4°, the fine precipitate was recovered by centrifugation (supernatant liquid discarded), resuspended in 5–10 ml. water and dialysed (48 hr., 0–4°) against 0·1 M-NaCl + 0·001 M-phosphate buffer (pH 6·8). The dialysed fluid was centrifuged (2000 *g*; 10 min.; 0–4°) and the residue discarded.

Chromatography on hydroxyl apatite. This procedure was carried out at 0–4°. A column (10 cm. × 1·6 cm.) of hydroxyl apatite was prepared and washed well with a solution containing 0·1 M-NaCl + 0·001 M-phosphate buffer (pH 6·8). The enzyme solution was passed through the column which was then washed with about 20 ml. of the above phosphate saline solution. The effluent was discarded and the column developed with a gradient from 0·1 M-NaCl + 0·001 M-phosphate buffer (pH 6·8) to 0·1 M-NaCl + 0·25 M-phosphate buffer (pH 6·8). The conditions of the gradient were described previously (see Drake, 1955, p. 18, case 4 with $k = 2$ and $V1 = 40$ ml.). Under these conditions, the neuraminidase began to elute when about 40 ml. had passed through the column. The eluate was collected in a fraction collector (1·3–2·0 ml. per tube) and the protein concentration in each tube estimated by measuring the absorption at 280 mμ. When haemolysis in the preceding stage had been slight, one sharp protein peak showing biological activity was obtained. The enzyme content of samples containing protein was estimated. Those samples which showed close agreement between enzyme activity and protein content (see Fig. 1*a*) were pooled. When more than one protein peak was present or when the neuraminidase peak trailed very badly, the samples containing most of the enzyme were pooled and again chromatographed as above. In either case, the appropriate tubes were pooled, dialysed against distilled water (48 hr.; 0–4°) and dried from the frozen state *in vacuo*.

Crystallization of neuraminidase. This procedure was carried out at 0–4°. The dried material was dissolved in 1–1·5 ml. water and dilute acetic acid added to pH 5·0. If the solution became opaque, it was clarified by centrifugation. (If too much acetic acid was added, the solution was neutralized with dilute NH_4OH, again dialysed and dried.) The solution was then cautiously brought to about pH 4·5 and rocked gently for 2–3 days. Crystallization was usually achieved by seeding; it was complete after 3 days. The crystals were then washed with water saturated with CO_2 and stored in water at 0–4°. They were dissolved by the addition of 0·01 N-NH_4OH.

RESULTS

Production of neuraminidase

Maximum production of neuraminidase in the culture medium under the conditions described usually occurred within 10 hr. In a typical experiment, the titre of enzyme (per 0·25 ml., estimated by the biological method) at different times after the inoculation of the medium was: 6 hr., 100; 7·5 hr., 350; 8·5 hr., 1300; 9·5 hr., 2400. After heating to kill the organisms, this fluid had a titre of 2200. In this experiment, most of the sialyl lactose present in the original medium (as estimated by the direct Ehrlich reaction) had been destroyed by 10 hr. (see French & Ada, 1959). Crude extracts from which crystalline neuraminidase has been obtained possessed titres (per 0·25 ml.) ranging from 1100 to 4000.

Table 1. *Purification of neuraminidase of* Vibrio cholerae

Procedure	Volume (ml.)	Activity* (units/ 0·25 ml.) ($\times 10^{-3}$)	Total units ($\times 10^{-6}$)	Protein† (mg/ml.)	Activity (units/ mg.)	Yield (%)	Purification
Crude extract	35,000	4	560	5·5	2·9	100	—
1. Methanol fractionation	810	160	520	5·5	116	93	40
2. Human red cell eluate	800	110	352	0·3	1,470	63	500
3. Ammonium sulphate fractionation	11·1	3,200	142	2·7	4,730	26	1,630
4. Chromatography on hydroxyl apatite	8·0	4,800	154	2·15	8,940	28	3,080
5. Crystallization	1·2	24,000	115	6·0	16,000	21	5,500

* Estimated by the biological method. At each stage during the purification procedure, samples were diluted 1/100 in calcium acetate broth and stored at −20°. All enzyme activities were determined on the same day.

† Determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Evaluation of purification procedure

The detailed analysis of the purification procedure as applied to one crude filtrate of a *Vibrio cholerae* culture is presented in Table 1. Measurements of the volume, enzyme activity and protein content of each fraction allowed an estimate to be made of the yield of pure enzyme and the degree of purification achieved. The major part of the overall purification achieved occurred in the first three steps (methanol fractionation, adsorption to and elution from red cells, ammonium sulphate fractionation). However, the recovery of enzyme was often poor at one or the other of these stages. In the experiment quoted, the major loss of activity occurred during the ammonium sulphate fractionation. Sometimes the greatest loss occurred at the preceding stage of adsorption to and elution from red cells. The low recovery often encountered here was not due to destruction of enzyme; the remaining portion could be accounted for in the fluid after extraction with red cells. In some experiments, a higher recovery of enzyme in the eluate was obtained by increasing greatly the number of red cells used as absorbent.

Chromatography of the ammonium sulphate precipitate on columns of hydroxyl apatite showed a single protein peak with a very steep leading edge, but the trailing edge varied in steepness from batch to batch. Figure 1*a* shows the pattern of enzyme activity and protein concentration given by the preparation quoted in Table 1. The close correspondence between enzymic activity (estimated by the biological method) and protein concentration seen at all stages of the peak except the tail end was fairly typical. A protein peak showing a more pronounced trailing edge and thus a greater disparity between protein concentration and enzymic activity was seen occasionally. For example, such a pattern was shown by a preparation in which substantial haemolysis had occurred during elution from red cells. In this case, the most active fractions were pooled and again chromatographed, then yielding a pattern like that shown in Fig. 1*b*.

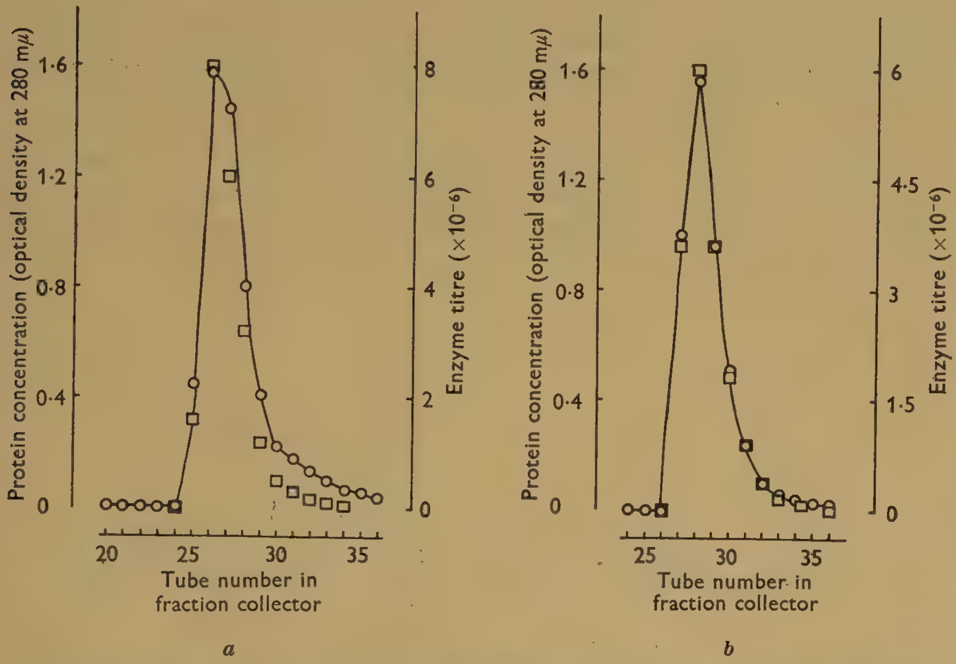


Fig. 1. Purification of *Vibrio cholerae* neuraminidase by chromatography on hydroxyl apatite. The elution pattern was obtaned by using a concentration gradient of phosphate buffer (pH 6·8). (*a*) First chromatographic run. (*b*) Re-chromatography of pooled selected fractions from first run. Protein concentration, ○—○; enzyme titre, □—□.

Coloured impurities were removed from the preparation at the chromatography stage so that crystallization was effected from a colourless solution. Crystallization completed the purification procedure. In the experiment quoted, the overall result was a decrease in volume from 35 l. to 1·2 ml., a yield of 20 % of the original activity as crystals and a 5500-fold purification. Nine batches of crystalline enzyme have been prepared. In six batches, the recovery was estimated and found to vary between 33 and 7 % with a mean value of 21 %.

One batch of enzyme behaved differently. The procedure applied differed in one respect from that described above in that the product of methanol fractionation was stored at −20° for 14 days. The crude extract had a very high titre (7500), but extensive losses were sustained at each stage of the purification procedure, the overall recovery at the ammonium sulphate stage being only 7 %. Chromatography on hydroxyl apatite showed a very broad protein peak. No attempt was made to obtain crystals from this batch.

Properties of neuraminidase

Neuraminidase crystallizes as needles (Pl. 1). Fig. 2 shows the ultraviolet absorption spectrum of the pure enzyme. $E^{1\%}_{280\,\mathrm{m}\mu}$ was 8·96, measured at 0·025 % (w/v). Application of the method of Lowry *et al.* (1951) for protein estimation to a solution of the enzyme (0·0152 %, w/v) yielded an optical density (0·5 cm. optical cell) of 0·194 at 750 mμ. The pure enzyme had a biological activity of $12{\cdot}6 \times 10^6$ units/mg. dry weight.

Failure to detect proteolytic activity. Crystalline enzyme (20 μg.) failed to show any sign of proteolytic activity against casein at pH 6·5; the test could have detected the presence of 0·02 μg. trypsin.

Failure to detect N-*ANA-aldolase activity.* Samples of *N*-acetylneuraminic acid (*N*-ANA; 3·4 mg.) were incubated with buffer or with buffer containing 20 μg. neuraminidase. After 24 hr., less than 15 μg. of *N*-acetylmannosamine was detected in either reaction mixture. This corresponds to less than 1 % conversion of *N*-ANA to *N*-acetylmannosamine.

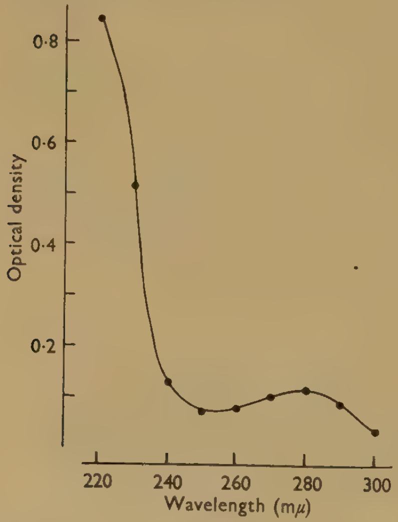


Fig. 2

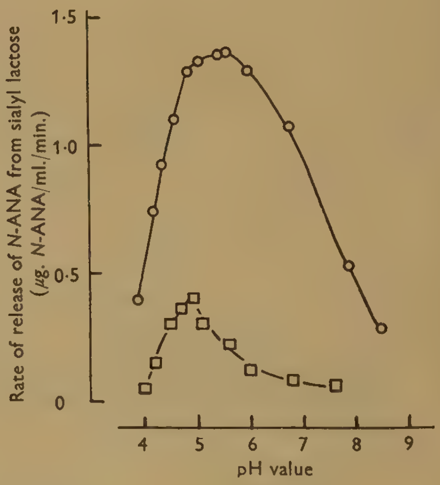


Fig. 3

Fig. 2. Ultraviolet absorption spectrum of *Vibrio cholerae* neuraminidase. Neuraminidase was dissolved in 0·08 M-phosphate buffer (pH 6·8). Concentration of enzyme, 0·025 % (w/v).

Fig. 3. The activity of *Vibrio cholerae* neuraminidase at different pH values. Composition of medium: sialyl lactose, 0·17 % (w/v); buffer 0·3 ml., prepared by mixing 0·1 M-tris + 0·1-M maleic acid; neuraminidase, 0·15 μg./ml.; total volume, 0·4 ml. □—□, no added $CaCl_2$; ○—○, added $CaCl_2$ (0·001 M).

Kinetic studies

Experiments were first carried out to determine the pH value at which optimum enzyme activity of purified enzyme preparations was shown. The suspension of enzyme crystals (0·025 ml.) was dissolved in 0·225 ml. 0·01 N-NH_4OH and the volume brought to 1 ml. by addition of tris + maleate buffer (pH 4·9). A further dilution into buffers of different pH values was made shortly before the start of the experiment. Under these conditions, the enzyme showed a maximum activity at pH 4·9 (Fig. 3, lower curve).

Influence of cations on the activity of neuraminidase. Calcium ions were found to stimulate enzyme activity. The results of a typical experiment are shown in Fig. 4, in which the effect of addition of different concentrations of $CaCl_2$ to the enzyme + substrate solutions is illustrated. Maximum stimulation was found at 0·001 M-$CaCl_2$. Stimulation was not observed when the $CaCl_2$ concentration was 0·1 M.

Enzyme activity was also affected by certain other cations. The chlorides of manganese, magnesium, barium, zinc or sodium were added to the medium to a final concentration of 0·001 M. The rate of release of *N*-ANA from sialyl lactose at pH 4·9 (expressed as μg. *N*-ANA/ml./min.) was in the presence of $CaCl_2$, 2·0; $MnCl_2$, 1·0; $ZnCl_2$, 0·7; $MgCl_2$, 0·4; $BaCl_2$ and NaCl, 0·3.

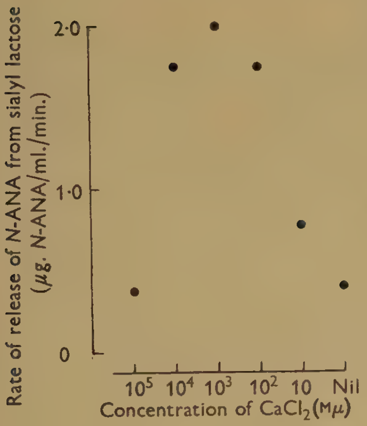


Fig. 4

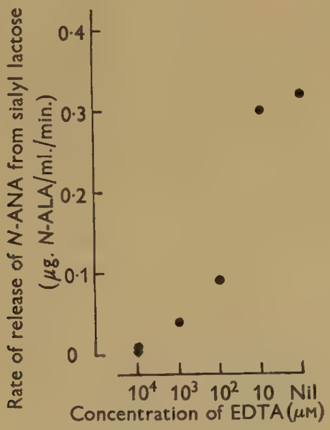


Fig. 5

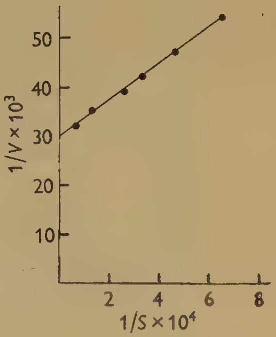


Fig. 6

Fig. 4. The activity of *Vibrio cholerae* neuraminidase in presence of added $CaCl_2$. Composition of medium: sialyl lactose, 0·17 % (w/v); tris + maleate buffer, 0·06 M (pH 5·6); neuraminidase, 0·27 μg./ml.

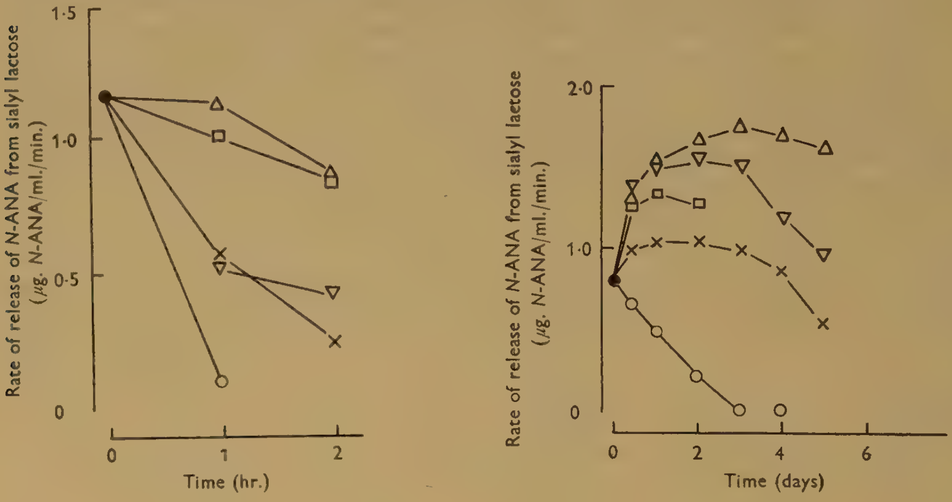
Fig. 5. The activity of *Vibrio cholerae* neuraminidase in the presence of added ethylenediaminetetra-acetate (EDTA). Composition of medium: sialyl lactose, 0·17 % (w/v); tris + maleate buffer, 0·06 M (pH 6·6); neuraminidase, 0·27 μg./ml.

Fig. 6. Activity of *Vibrio cholerae* neuraminidase as a function of substrate concentration. S is the concentration (M) of substrate; V is the velocity of action (plotted on an arbitrary scale).

Inhibition of enzyme activity by ethylenediamine tetra-acetate. In Fig. 5 are plotted the results of one of two experiments to investigate the effect of added EDTA on the activity of neuraminidase. Increasing inhibition of enzyme activity occurred as the concentration of EDTA was raised. In a separate experiment, enzyme (75 μg./ml.) was stored in 0·01 M-EDTA (pH 5·6) for 40 hr. at 0–4°. It was then diluted and tested in the presence of 0·001 M-$CaCl_2$ and showed full activity.

Effect of pH *value on neuraminidase activity.* In the presence of 0·001 M-$CaCl_2$ neuraminidase showed maximum activity at pH 5·6. Below pH 5 and above pH 6, the activity decreased rapidly (Fig. 3, upper curve).

Enzyme constants. Constants were determined at 37·1° in tris + maleate buffer (pH 5·6; 0·07 M) containing $CaCl_2$ (0·001 M), bovine plasma albumin (0·03 %) and sialyl lactose. A Lineweaver & Burk (1934) plot of the data yielded a value of $1{\cdot}2 \times 10^{-3}$ M for the Michaelis constant K_m (Fig. 6). At an enzyme concentration of 0·16 μg./ml., $V_{\text{max.}}$ was 0·021 μM-*N*-ANA/min./ml.



a *b*

Fig. 7. Stability of *Vibrio cholerae* neuraminidase stored at (*a*) different pH values at 37° and (*b*) at 0–4°. The activity of the stored enzyme (about 15 μg./ml.) was determined in the standard way at pH 5·6 and in the presence of 0·001 M-$CaCl_2$. pH 8·5, ▽; pH 6·7, △; pH 5·6, □; pH 4·6, ×; pH 3·9, ○.

Stability of purified neuraminidase

The stability of neuraminidase in solution was tested at 37° and 0–4°. Some tests were also carried out on the dry enzyme.

Stability at 37°. Neuraminidase lost activity when incubated at 37°. This was first noticed when estimating the velocity in the chemical test for enzyme activity. For example, the decrease in velocity per min. at different pH values was as follows: pH 4·3, 0·45 %; pH 5·0, 0·4 %; pH 6·2, 0·3 %; pH 7·9, 0·2 %.

To find the pH value of optimum stability, some crystals were dissolved in NH_4OH and brought to about pH 5·6. Samples (0·1 ml.) were added to 0·9 ml. volumes of buffer at pH 3·9, 4·6, 5·6, 6·7 and 8·5 (final enzyme concentration about 15 μg./ml.). These mixtures were then incubated at 37° for 1 hr.; some of the pH 5·6 sample was kept at 0°: Samples from each mixture were then added to a solution of sialyl lactose and $CaCl_2$ buffered at pH 5·6 and the enzyme activities determined. The enzyme solutions, incubated at 37°, had the following activities expressed as a percentage of the control solution kept at 0°: pH 3·9, 9 %; pH 4·6, 50 %; pH 5·6, 112 %; pH 6·7, 130 %; pH 8·5, 42 %. In a second experiment, dissolved enzyme was stored at 0–4° (pH 5·6) for 18 hr. before incubation at 37°. The results of this experiment are plotted in Fig. 7*a*. The enzyme was most stable in the range pH 5·6–6·7, about 24 % of the activity being lost in 2 hr. The enzyme was particularly unstable at pH 3·9.

Stability at 0–4°. The experimental procedure followed here was similar to that described in the preceding paragraph except that the enzyme solutions (at pH 3·9, 4·6, 5·6, 6·7, 8·5) were held at 0–4° for several weeks. At intervals samples were removed and the enzymic activity determined at pH 5·6. The results are plotted in Fig. 7*b*. The most striking feature was the rise in activity shown by enzyme stored at the higher pH values. After this initial rise, the activity again slowly decreased.

Stability of dried enzyme. Purified enzyme to be sent to other laboratories was dried from the frozen state in glass ampoules which were then sealed under vacuum. When stored at 0–4°, such material retained nearly full activity (as tested by the biological method) for at least 6 months.

DISCUSSION

Purification procedure. Several factors have contributed to the successful outcome of this work. In the first place the presence in the medium of sialyl lactose, an inducer of neuraminidase (French & Ada, 1959), ensured the production of high titres of enzymes. The crude extract thus had a high specific activity (enzyme units/mg. protein). Secondly, the medium contained only simple ingredients which were not precipitated by 60 % (v/v) methanol in water at 0° and this largely accounted for the 40-fold purification achieved in the first step. In addition, the enzyme was concentrated about 40-fold in this step and thus was contained in a volume small enough to allow rapid extraction with red cells, by using ordinary laboratory facilities.

The process of specific adsorption to and elution from red cells, first introduced by Burnet & Stone (1947), played an important part in the purification procedure. The recovery rate of this step was about 50 %. Attempts to increase the yield of enzyme in the eluate were unsuccessful; increasing the number of red cells used as adsorbent did not always result in improved yields. The use of ethylenediamine-tetra-acetic acid at room temperature to effect rapid elution of the enzyme, instead of prolonged incubation of enzyme and red cells at 37° (Ada & French, 1950), decreased the contamination of the eluate with red cell breakdown products.

Precipitation of the enzyme with ammonium sulphate was a convenient way of decreasing the volume to be handled and of restoring the protein concentration to a high value which was maintained in the later stages of the process. Although not formally demonstrated, our experience was consistent with the belief that removal of most of the impurities by chromatography on hydroxyl apatite was a prerequisite for a high yield of crystalline enzyme. While the yield of crystalline enzyme was satisfactory there were two stages in the procedure where substantial losses occurred. Experiments in progress may show ways of decreasing these losses.

Properties of the enzyme. No information about the chemical properties of the enzyme is yet available. A preliminary examination of the physical properties is reported in the succeeding paper (Pye & Curtain, 1961). The results suggested that the crystalline enzyme was homogeneous by the usual physical criteria.

Sialyl lactose was used as the substrate in kinetic studies for several reasons. It is a well characterized compound, is readily prepared in large amounts, and since it is very soluble, high substrate concentrations can be achieved. However, kinetic constants may vary with the substrate used.

The addition of Ca (0·001 M) to the reaction medium stimulated neuraminidase activity (cf. Burnet & Stone, 1947). High concentrations (0·1 M) of Ca did not stimulate activity, a feature which has been noticed with other metal + enzyme systems. The addition of Ca ion to the medium changed the shape of the pH–activity curve; the value for optimum activity was displaced from pH 4·9 to pH 5·6. The mode of action of added Ca ion (whether it combined with enzyme or substrate or acted as a link beween enzyme or substrate) remains to be investigated. The activity shown by neuraminidase in the absence of added Ca ion was inhibited by ethylenediaminetetra-acetic acid which suggests that the enzyme as isolated contained a metal as an essential component.

A puzzling feature of the stability results was the initial increase in activity on storage of the enzyme, particularly at 0–4°. Here the rise was observed at all pH values except 3·9 and was greater as the pH value increased. At the higher values tested (pH 6·7, 8·5) this increase occurred over 3 weeks, reaching a value about double the original. This effect was less clear at 37°. It seems likely that two opposing factors were operating: one was a change of the enzyme to a more active form (disaggregation?), a process favoured by storage of dilute solutions at high pH values, and the other was thermal inactivation of the enzyme. This finding means that the calculated values for K_m and $V_{\text{max.}}$ should be regarded as approximate rather than absolute. It should also be pointed out that, despite the variation in thermal stability shown by enzyme stored at different pH values, the curve in Fig. 3 is essentially a true indication of the activity of the enzyme at each pH value, since velocity values were obtained by extrapolation to zero time.

Schramm & Mohr (1959) independently published a method for the purification of neuraminidase from *Vibrio cholerae* cultures. The properties of their preparation are different from those described here. In addition to the low molecular weight (10,000–20,000) they found their preparation was very unstable unless stored in the presence of chelating agents or cyanide. The K_m value of their preparation ($1–2 \times 10^{-4}$ M) is different from that found with our preparation ($1·2 \times 10^{-3}$ M) but this might be due to the smaller enzyme molecule and the different substrate used by Schramm & Mohr (sheep red cells). We cannot as yet offer any experimental evidence which would explain the many differences between the two preparations.

We wish to thank the different organizations, particularly the Victorian Department of Agriculture, who have supplied us with bovine colostrum. The work was supported by the National Health and Medical Research Council, Canberra, Australia. The authors are also indebted to Mr K. Clarke for excellent technical assistance.

G. L. ADA, E. L. FRENCH AND P. E. LIND (*Facing p.* 421)

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EXPLANATION OF PLATE

Crystalline neuraminidase (phase contrast; × 300). Photograph by Mr E. Matthaei.

J. gen. Microbiol. (1961), 24, 423–425
With 1 *plate*
Printed in Great Britain

Electrophoretic, Sedimentation and Diffusion Behaviour of Crystalline Neuraminidase from *Vibrio cholerae*

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(*Received* 21 *October* 1960)

SUMMARY

Crystalline neuraminidase prepared from *Vibrio cholerae* culture fluids by Ada, French & Lind (1961) was examined by moving boundary electrophoresis, analytical ultracentrifugation and diffusion. The various corresponding physical constants were obtained. The results indicated that, within the limits of the methods used, the crystalline protein was enzyme and was homogeneous.

METHODS

Neuraminidase. Crystalline neuraminidase (a pool of three batches) was supplied by Dr G. L. Ada. Because of the scarcity of the purified enzyme, all physical measurements were carried out on this pool. The enzyme was submitted first to diffusion analysis, then to electrophoresis and finally to sedimentation in the ultracentrifuge. After each examination, the enzyme was dialysed against distilled water and concentrated by sublimation of water from the frozen state *in vacuo*. In some cases, a considerable time elapsed between experiments and during these periods the dissolved enzyme was stored at −20°.

Buffers. For electrophoresis: (1) sodium veronal (pH 8·5, 0·012 M) + sodium chloride (0·09 M); (2) sodium phosphate (pH 6·7, 0·0056 M) + sodium chloride (0·09 M); (3) sodium acetate (pH 5·1, 0·0145 M) + sodium chloride (0·09 M). The ionic strength of each of these buffers is 0·1.

For sedimentation: (4) sodium veronal (pH 8·5, 0·01 M) + sodium chloride (0·1 M); (5) sodium phosphate (pH 6·7, 0·01 M) + sodium chloride (0·1 M).

For diffusion: the sodium phosphate buffer (no. 5) used for sedimentation.

Moving boundary electrophoresis. This was carried out at 4° in a Tiselius type equipment with cylindrical lens schlieren optical system. A 2 ml. optical cell was used. The assembly was closed on the anode side. At the conclusion of each run the cell was closed and the contents of the two limbs and the base removed separately for biological assay of enzyme activity (French & Ada, 1959). We are indebted to Dr P. E. Lind for carrying out these estimations. Mobilities of the boundaries observed and of the enzyme activities were calculated for the descending limb by using the conductivities of the supernatant buffer solutions.

Ultracentrifugation. An air-driven Beams-Pickels type of centrifuge with a cylindrical lens schlieren optical system was used at a rotor speed of 800 rev./sec. and a temperature of about 20°.

Diffusion. The diffusion experiment was carried out at 1·5° in a 2 ml. cell in the Perkin–Elmer Model 38 A electrophoresis apparatus with the phase-plate gradient optical system of Moore & Opperman (1956). The cell bath of this instrument was maintained to ±0·02° by circulating water from a refrigerated tank (built by Cordley & Hayes, New York, for the Perkin–Elmer Corporation).

RESULTS

Electrophoresis. The electrophoretic patterns observed at pH 6·7, 8·5 and 5·1 are shown in Pl. 1, fig. 1(*a*), (*b*), (*c*). In each case a single peak was observed which migrated towards the anode. In the first two analyses the peaks were asymmetrical. During dialysis against the pH 5·1 buffer, a slight precipitate formed and this was removed by centrifugation. The peak subsequently observed during electrophoresis at pH 5·1 was almost symmetrical. The mobilities of these boundaries and of the enzyme activities are given in Table 1.

Table 1. *Electrophoretic mobilities of* Vibrio cholerae *neuraminidase at various* pH *values*

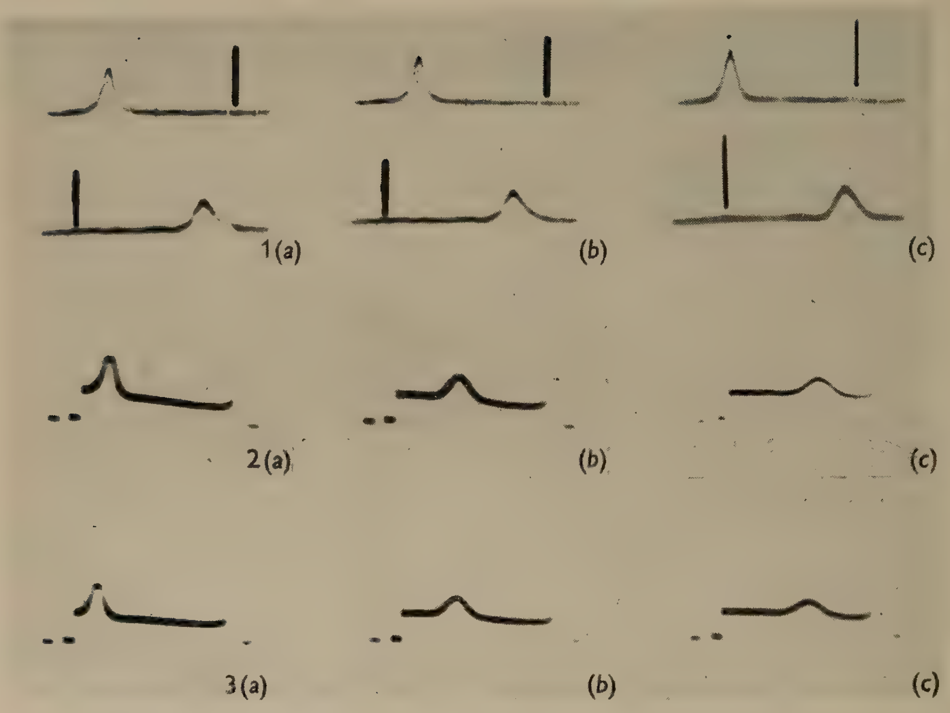
pH value:		6·7	8·5	5·1
Mobilities (10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1})	Protein boundary:	3·8	3·9	2·1
	Enzyme activity:	3·8	4·1	2·1

Sedimentation. The enzyme sedimented as a single symmetrical boundary at pH 6·7 and 8·5 (see Pl. 1, fig. 2 (*a*), (*b*), (*c*) and fig. 3 (*a*), (*b*), (*c*)). At pH 6·7, the sedimentation was observed at concentrations of 0·9 %, 0·6 % and 0·3 % (w/v). There was no apparent dependence of sedimentation coefficient on protein concentration within the accuracy of the methods used. At pH 6·7, s_{20} was 5·5 S; at pH 8·5, 5·3 S.

Diffusion. At a concentration of 0·72 % (w/v) and pH 6·7 the *Vibrio cholerae* neuraminidase gave a value for D_{20} of $5{\cdot}5 \times 10^{-7}$ cm.^{2}sec.$^{-1}$.

DISCUSSION

The results of the sedimentation and electrophoresis measurements left little doubt that we were dealing with a protein preparation which was substantially homogeneous, at least within the limits of the methods used. The small amount of material insoluble at pH 5·1 might have been denatured or aggregated neuraminidase and as such could have been responsible for the asymmetry of the electrophoretic boundaries at pH 6·7 and 8·5. The agreement between the electrophoretic mobilities of the enzymically active material and the protein boundaries at three pH values suggested that the crystalline protein was in fact neuraminidase. This was supported by the finding that a single peak with constant specific activity was obtained during chromatography of the material on hydroxy-apatite (Ada *et al.* 1961). No attempt was made to correlate the sedimenting boundaries with enzymic activity. The mobility values indicated that the isoelectric point of neuraminidase



J. PYE AND C. C. CURTAIN

was below pH 5·1. The instability of the enzyme at lower pH values (Ada *et al.* 1961) precluded any attempt to determine the actual value.

If a partial specific volume of 0·75 be assumed, calculation from the sedimentation and diffusion coefficients yields a molecular weight of 90,000 and a frictional ratio (f/f_0) of $1{\cdot}2_0$. These results were obtained with a phosphate buffer at pH 6·7; the sedimentation coefficient in veronal buffer at pH 8·5 was not significantly different. Schramm & Mohr (1959) obtained a sedimentation coefficient of 1·3 S in veronal buffer at pH 8·5 in presence of potassium cyanide for a neuraminidase isolated from filtrates of *Vibrio cholerae* cultures; they found evidence of aggregation in other media (unspecified). There are at present insufficient data to account for the differences between the two enzyme preparations.

We are grateful to Mr H. F. Holden who carried out the sedimentation runs and to Mr I. J. O'Donnell for the use of a Cambridge Universal Measuring Machine. One of us (J.P.) was supported by the National Health and Medical Research Council, Canberra, Australia.

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EXPLANATION OF PLATE

Fig. 1. Moving boundary electrophoresis of *Vibrio cholerae* neuraminidase. (*a*) pH 6·7; 5·8 volt cm.$^{-1}$ for 189 min. (*b*) pH 8·5; 5·7 volt cm.$^{-1}$ for 177 min. (*c*) pH 5·1; 5·6 volt cm.$^{-1}$ for 327 min. In each case the upper and lower patterns represent the ascending and descending limbs, respectively. The vertical line indicates the initial position of the boundary.

Fig. 2. Sedimentation of *Vibrio cholerae* neuraminidase. 0·9 % (w/v) at pH 6·7. Speed of rotor, 800 rev./sec. From left to right, exposures at 43 (*a*), at 111 (*b*) and at 164 min. (*c*) after reaching full speed.

Fig. 3. Sedimentation of *Vibrio cholerae* neuraminidase. 0·8 % (w/v) at pH 8·5. Speed of rotor 800 rev./sec. From left to right, exposures at 37 (*a*), at 97 (*b*) and at 157 min. (*c*) after reaching full speed. In every case sedimentation occurs from left to right.

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Morphological Variation in *Spirillum* spp., with Observations upon the Origin of the Hyphomicrobia

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(*Received* 2 *November* 1960)

SUMMARY

Pure cultures of *Spirillum serpens* grown on solid medium contained variants of very diverse morphological appearance; these were relatively straight rods, and branched coccobacillary and coccal forms. Flagella were in the normal lophotrichous arrangement, peritrichous, single or absent. Some forms possessed characters resembling those of the hyphomicrobia. The development of Rhodomicrobium in cultures of photosynthetic spirilla was also observed. It is suggested, not only that the spirilla possess potentialities consistent with a suggested role as the ancestral bacterial type, but also that Rhodomicrobium may be a growth form of a photosynthetic spirillum. Appearances found in the parasitic *Hyphomonas polymorpha* and in certain pleuropneumonia-like organisms were considered to support this concept.

INTRODUCTION

It was suggested by Bisset (1950) that the spirilla probably conform most closely to the ancestral bacterial type, and that many of the morphologically distinct types considered to have arisen from them may be regarded as adaptations to a terrestrial existence, a process paralleled in many natural groups. In support of this idea, the present paper describes a variety of morphological forms adopted by a single species of spirillum (*Spirillum serpens*) when cultivated on the surface of solid medium. These forms simulate many of the major characters of the hypothetically more advanced bacteria, and indicate a potential, in spirilla, for the production of such characters, in accordance with the evolutionary theory in question. The existence of a complex life-cycle in spirilla is well attested (e.g. Pease, 1956*a*; Watanabe, 1959, 1960; and others). Certain of the appearances here described are attributable to phases in such a cycle, others are probably not. At the same time, some light may be thrown by these observations upon the problem of the origin and nature of the hyphomicrobia; of these three genera are recorded, each of one species, variously classified by different authorities, possessing a similar and very unusual morphology, and being credited with great versatility in their modes of life. The first descriptions of *Hyphomicrobium vulgare* attributed to it the power of fixing nitrogen (Rullman, 1897; Stutzer & Hartleb, 1899), although it is now regarded as an oligotrophic saprophyte. *Rhodomicrobium vannielii* is considered to be a photosynthetic purple sulphur bacterium (Duchow & Douglas, 1949). *Hyphomonas polymorpha* was isolated in circumstances suggestive of pathogenicity towards man (Pongratz, 1957). All these micro-organisms are distinguished by their morphology and mode of growth, which is quite unlike that of the recognizable forms of other bacteria, in that the cells are borne upon narrow, often ramifying filaments, and reproduce by the outgrowth of similar filaments, upon which small buds appear and grow up into new cells. The cytology of this process was described by Murray & Douglas (1950), but the mechanism cannot be regarded as well understood. Despite the stalk-like filaments, this mode of reproduction is unlike that of the true caulobacteria, which undergo fission in a relatively normal manner (Bisset, 1955, pp. 121–4). Although true motility is possible only in small detached elements, flagella are commonly borne upon various parts of the ramifying growth, in all recognized genera. The attachment is described as polar in *Hyphomicrobium vulgare* (Mevius, 1953) and *Hyphomonas polymorpha* (Pongratz, 1957), while Douglas & Wolfe (1959) considered the flagellation of *Rhodomicrobium vannielii* to be peritrichous; but the electron micrographs of Pease (1956*b*) show polar attachment in *R. vannielii.* Our own view is that the normal criteria of flagellar insertion must be applied with caution to micro-organisms of this morphology.

The circumstances in which Hyphomicrobium and Rhodomicrobium are usually observed are of importance in any consideration of their possible nature and relationships. They arise, in a manner for which no real explanation has hitherto been offered, in ageing and degenerate cultures of other bacteria, especially nitrifying bacteria in the first case, and photosynthetic spirilla in the second. Some strains, arising in this manner, can be isolated and subcultured, others appear to grow freely only in mixed culture.

METHODS

Observations were made upon a strain of *Spirillum serpens* (provided by Dr Phyllis Pease) which had been isolated from pond water and maintained in pure culture for 6 years; also upon four strains of *Rhodomicrobium vannielii*, observed in ageing cultures of photosynthetic spirilla, and two of *Hyphomonas polymorpha* provided by Dr E. Pongratz, by whom they were isolated from a nasal discharge.

The medium used was similar to that of Duchow & Douglas (1949); its composition (%, w/v) was: yeast extract (Oxoid), 0·2; sodium acetate, 0·2; sodium citrate, 0·2; ammonium sulphate, 0·1; magnesium sulphate, 0·01; di-potassium hydrogen phosphate, 0·05; sodium chloride, 0·2; at pH 7·2, solidified with 2 % (w/v) agar; in Petri dishes for *Spirillum serpens*, and dispensed in small screw-capped bottles with caps tightly fastened, for the photosynthetic forms. The organisms used grew at room temperature; *Hyphomonas polymorpha* grew on meat infusion agar at 37°.

Electron micrographs were made upon material taken directly from fluid cultures and dried upon collodion films, or from suspensions in distilled water in the case of growth on solid medium.

RESULTS

The morphology of *Spirillum serpens* in fluid culture, or on initial subculture on solid medium in Petri dishes, was very characteristic, consisting of spiral rods of moderate length, with a tuft of ten to fifteen flagella at each pole (Pl. 1, fig. 1). On continued subculture on solid medium the colonies lost their original smooth appearance and showed a rough spreading form. When microscopically examined, these rough colonies were seen to consist mainly of typical spirilla, but with about 20–30 % of other forms including relatively straight rods (Pl. 1, figs. 2, 4) and branched forms (Pl. 1, figs. 4, 5, 6). Flagellation was mainly of the normal polar type, but peritrichous arrangements were also seen, both in straight rods (Pl. 1, fig. 2) and in spirilla (Pl. 1, fig. 3). In the later stages of culture, after 7–10 days, typical minute swarm-cells began to appear (Pl. 1, fig. 7; cf. Pease, 1956*a*), some of which possessed the characteristic single polar flagellum, whereas other small rod-shaped organisms were non-flagellate (Pl. 2, figs. 8, 12). In association especially with the latter were two forms of unusual appearance: (*a*) spherical cells somewhat resembling free protoplasts, but with coiled flagella (Pl. 2, figs. 8, 9); (*b*) stalked cells (Pl. 2, figs. 11–13). The special interest of these lay in their resemblance to hyphomicrobia (compare Pl. 2, figs. 14, 15; Pl. 3, figs. 16–18). Another type of cell arising at this stage in cultures of *S. serpens* was not exceptional in itself, being a short oval, without flagella, but once more its resemblance to certain stages in the life-cycle of hyphomicrobia was most striking (Pl. 2, fig. 10; compare Pl. 2, fig. 15).

Rhodomicrobium vannielii was observed in the later stages of crude cultures ot photosynthetic spirilla (Pl. 2, figs. 14, 15). It began to appear in cultures aged 2–3 weeks, and gradually increased in proportion until the entire culture was of this form, consisting of oval cells joined by narrow branched filaments. Flagella were seen on occasional cells in most fields; they were very frequently coiled (Pl. 3, figs. 16–18). As these cultures aged, after 5–6 weeks or longer, both stalks and flagella became less obvious, and the bacteria often aggregated in stellate clumps (Plate 3, figs. 18, 19). In this condition they quite closely resembled those eubacteria, including spirilla (Watanabe, 1959, 1960), which adopt a similar habit, except for the appearance of an occasional stalk or projecting filament, or more rarely a coiled flagellum. Subculture from the earlier stages of culture usually gave a mixture of hyphomicrobia and other bacteria; but after the stellate phase had commenced, the hyphomicrobia were seldom detectable in subcultures until these had again aged.

The hyphomonas strains were distinguishable from Rhodomicrobium in that they branched much less freely, and produced mainly short irregular flagella (Pl. 3, fig. 20). The characteristic form was a globular cell attached by a slender filament to a smaller cell (presumably in course of development) at the other end. For comparison, observations were made upon growth forms of pleuropneumonia-like organisms, maintained in culture in this laboratory, some of which bore a distinct resemblance to hyphomicrobia (Pl. 3, fig. 21).

DISCUSSION

The theory of bacterial evolution proposed by Bisset (1950) and subsequently elaborated (Bisset, 1955), depends for its validity upon the potentialities of spirilla as forebears of all or most of the main groups of bacteria. The postulated development of different flagellar types from polar-flagellate aquatic ancestors appears to be acceptable to certain workers in this field (Leifson, 1960, p. 18) by whom the theory has been adopted. The observations reported in the present paper provide evidence that spirilla, when constrained to adopt an unnatural mode of existence on solid medium, may produce morphological forms which simulate, in certain respects, those bacteria whose morphology is believed to be an adaptation of the original spirillar form to a terrestrial environment. The appearance of straight or branched rods with typically spirillar flagella, or of peritrichous flagella upon spiral rods, may be regarded as indicating that all these variant characters are separately associable with the problem of adaptation to terrestrial environments. At the same time, the mixture of normal and variant characters in these organisms serves to confirm their identity with the original strain. This obviation of any suggestion of contamination is important also in consideration of the stalked forms, and others resembling hyphomicrobia, which occur in the latter stages of culture. The accepted theory of origin of hyphomicrobia is that they exist as contaminants in cultures of autotrophic bacteria, which they eventually overgrow. However, the existence of comparable forms in pure cultures of spirilla, and the peculiarities of the life-cycle of strains of Rhodomicrobium derived in the normal way from cultures of photosynthetic spirilla, render it at least equally probable that Rhodomicrobium also is a variant or growth form of a Rhodospirillum. These observations are not unique, in that Watanabe (1959) not only illustrates spirilla in stellate clusters of the appearance of those adopted by Rhodomicrobium, but also forms derived from the growth-cycle of saprophytic spirilla that bear a strong resemblance to hyphomicrobia. It is significant that hyphomicrobial morphology can be adopted by the parasitic *Hyphomonas polymorpha*, which also has flagella of degenerate appearance. The coiled flagella found in Rhodomicrobium and in the hyphomicrobial forms of *Spirillum serpens* occur in other bacterial groups (Leifson, 1960), but are especially distinctive of Rhodomicrobium. The resemblance already noted between hyphomicrobia and certain stages of pleuropneumonia-like organisms is demonstrated with even greater clarity in the figures of Ledingham (1933) and Turner (1935). It may perhaps be stated, without further elaborating the argument here, that these organisms are now accepted by some workers to be growth forms of true bacteria. Thus it would appear that the adoption of a morphology of this type is quite widespread among bacteria of different groups.

In view of the suggestion already made, in support of the evolutionary concept quoted at the beginning of this discussion, that the variant forms described here are adaptations to growth on solid medium, it is interesting to consider that the hyphomicrobial morphology is adapted to growth in attachment to solid surfaces, usually the sides of vessels containing fluid medium. Even the very small swarm cells, with their single polar flagella, are capable of motility in the thin film of moisture on an agar surface, where normal spirilla are usually completely immobilized. In appearance, these swarmers closely resembled vibrios, and although not strictly variants as in the case of the bacillary branched peritrichously-flagellate or hyphomicrobial forms, they afford further evidence of the ability of spirilla to produce or simulate most of the characters of the bacterial groups to which, on the hypothesis of Bisset (1950, 1955), they may be considered as ancestral.

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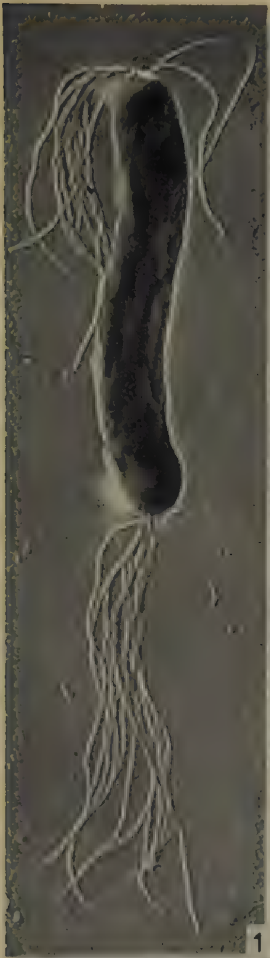

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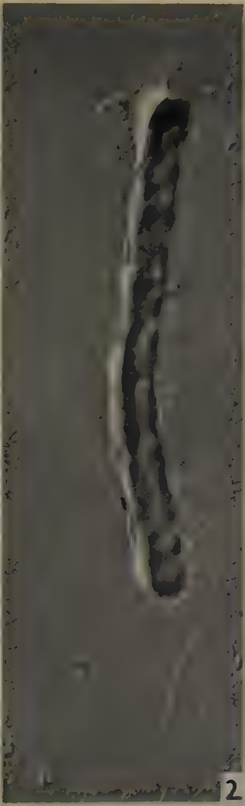

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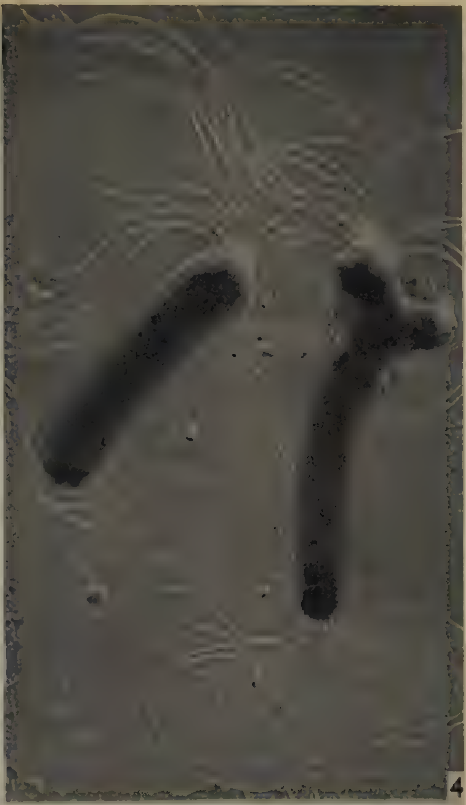

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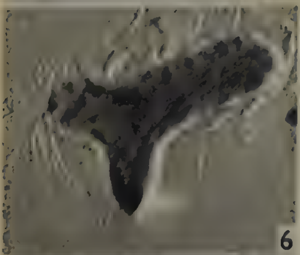

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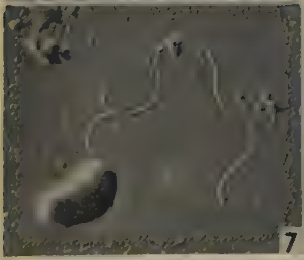

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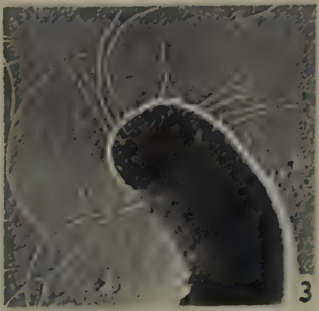

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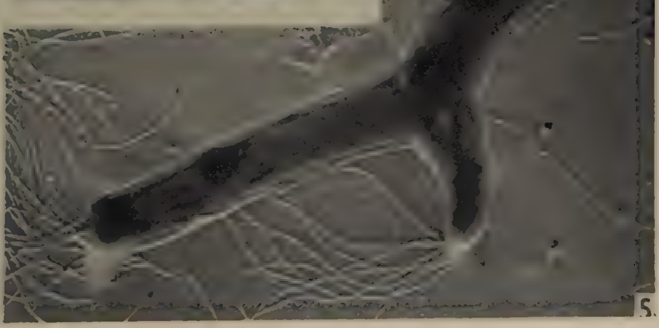

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K. A. BISSET

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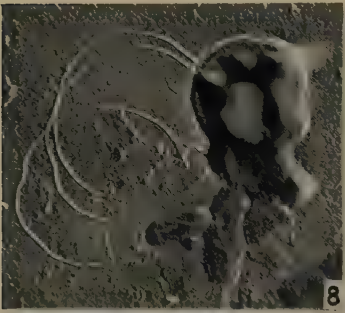

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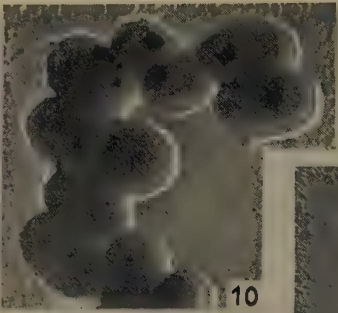

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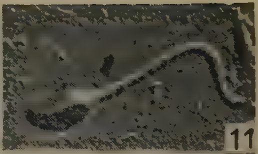

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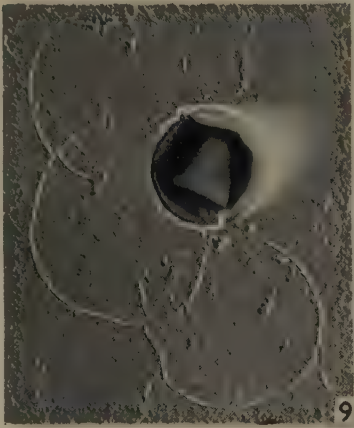

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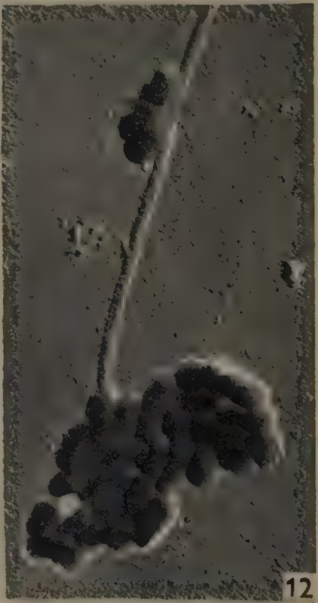

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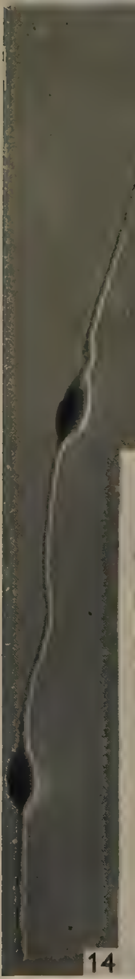

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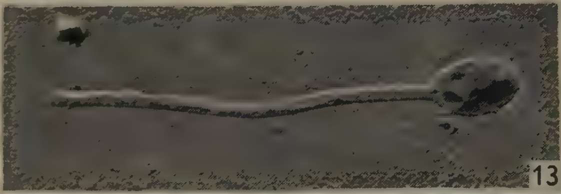

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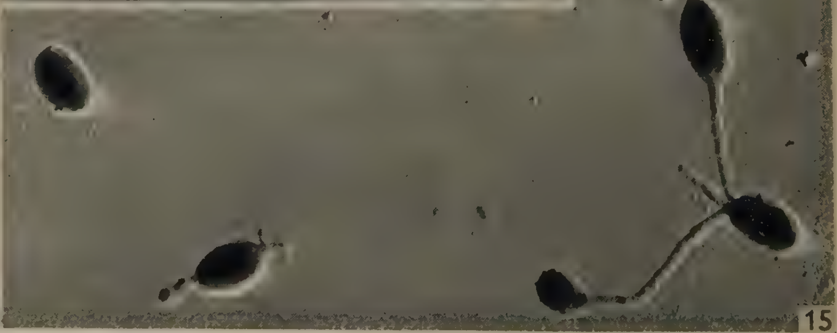

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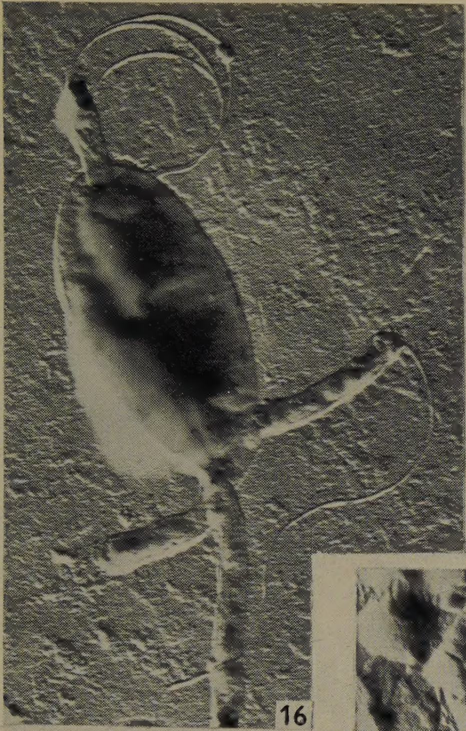
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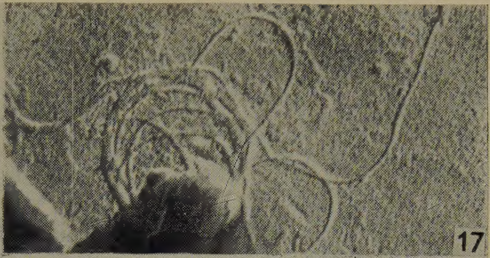
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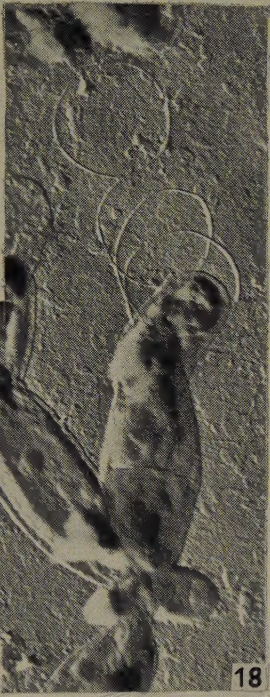
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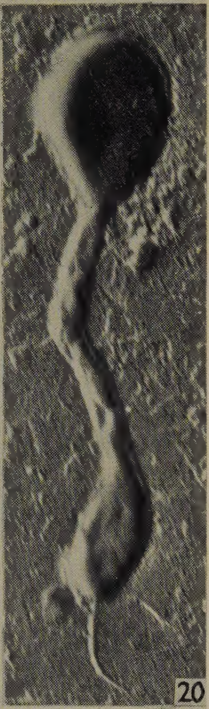
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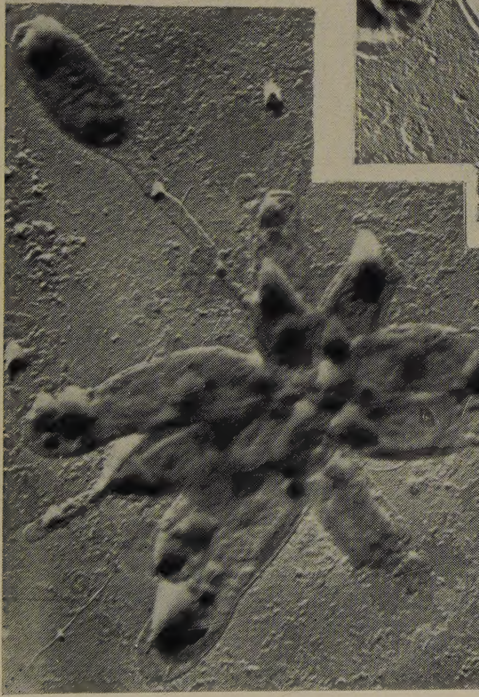
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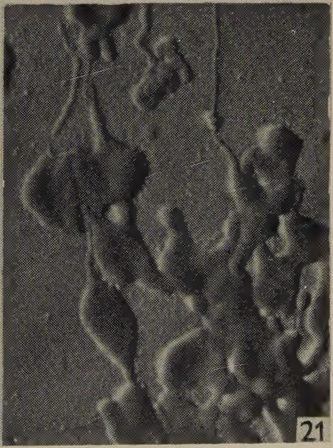
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EXPLANATION OF PLATES

(All figures are electron micrographs, gold-palladium shadowed.)

PLATE 1

Fig. 1. *Spirillum serpens:* normal appearance, spiral with polar flagella. ×10,000.

Fig. 2. *S. serpens:* rod form with irregular peritrichous flagella. ×10,000.

Fig. 3. *S. serpens:* spiral form with peritrichous flagella. ×10,000.

Fig. 4. *S. serpens:* straight and branched rods with polar flagella. ×10,000.

Figs. 5, 6. *S. serpens:* branched rods with polar flagella. ×10,000.

Fig. 7. *S. serpens:* swarmer cell, with single flagellum. ×10,000.

PLATE 2

Figs. 8, 9. *Spirillum serpens:* spherical cells (protoplasts?) associated with swarmers, showing coiled flagella. ×10,000.

Fig. 10. *S. serpens:* non-flagellate oval cells (compare fig. 15). ×10,000.

Figs. 11–13. *S. serpens:* stalked forms associated with swarmers (compare figs. 14, 15). ×10,000.

Figs. 14, 15. *Rhodomicrobium vannielii:* branched filaments and individual cells (compare figs. 10–13). ×7,500.

PLATE 3

Figs. 16, 17. *R. vannielii:* showing coiled flagella. ×20,000.

Figs. 18, 19. *R. vannielii:* stellate clumps, mainly bacillary forms, with occasional stalks and coiled flagella. ×10,000.

Fig. 20. *Hyphomonas polymorpha:* cell with short filament and bud bearing single, short flagellum. ×20,000.

Fig. 21. Organism of contagious agalactia (goat) showing hyphomicrobial forms. ×15,000.